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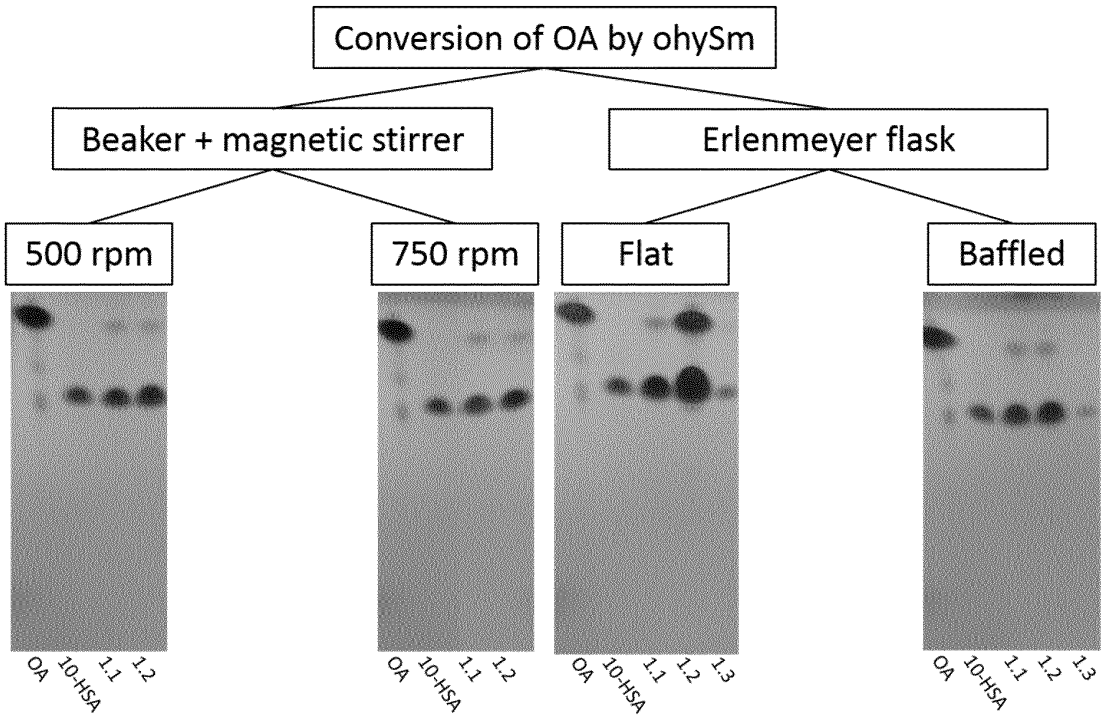
(54) **A PROCESS FOR THE CELL-FREE ENZYMATIC PRODUCTION OF 10-HYDROXYSTEARIC ACID (10-HSA) FROM BIO-BASED OILS FOR LUBRICANT FORMULATION**

(57) The present invention relates to an enzymatic process for the modification of free fatty acids (FFAs) derived from renewable feedstocks of bio-based oils. Specifically, the invention describes the hydrolysis of any bio-based oil, such as high oleic sunflower oil (HOSO), to FFAs, containing high amounts of oleic acid (OA), which is further hydrated to 10-hydroxystearic acid (10-HSA).

Figure 5:



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Description

Field of the invention

[0001] The present invention describes a method for the biological modification of natural, e.g., bio-based oils for lubricant/grease formulation. More particular, the invention describes an enzymatic process for the modification of free fatty acids (FFAs) derived from renewable feedstocks of bio-based oils. Specifically, the invention describes the hydrolysis of any bio-based oil, such as high oleic sunflower oil (HOSO), to FFAs, containing high amounts of oleic acid (OA), which is further hydrated to 10-hydroxystearic acid (10-HSA).

Background of the invention

[0002] Currently most additives used in lubricant or grease formulation are based on mineral oil feedstocks. Only a very low number of compounds used in lubricant production are from renewable feedstocks, for example 12-hydroxystearic acid isolated from castor oil.

[0003] The ability of different organisms to convert oleic acid to 10-hydroxystearic acid (10-HSA) was first described by Wallen et al.[1] in 1962 for the Pseudomonas strain 3266. The enzyme, responsible for this hydration reaction, was first isolated and characterized by Bevers et al.[2] in 2009 and defined as an oleate hydratase (EC 4.2.1.53). The oleate hydratase adds a hydroxyl group to the first position of the double bond in the chain of a mono unsaturated fatty acid in the absence of any co-factor that is consumed during the reaction (Scheme 3). A detailed description of the enzymatic reaction was published by Engleder et al. [3] in 2015. So far only two oleate hydratases have been crystalized [3-4] and most enzymatic reactions published only describe the production of 10-HAS from isolated oleic acid. At present, there are only two processes describing the conversion of complex oils to hydroxyl fatty acids [5]. However, these publications are lacking two essential steps, the separation of the 10-HSA from the reaction mixture and the recycling of the applied catalyst. Therefore, the objective of the present invention is to provide a process overcoming these shortcomings. The sustainable process described herein demonstrates the enzymatic conversion of vegetable oil (e.g., HO SO), as a renewable feedstock for bio-based oils, to 10-HSA with a product separation and an integrated enzyme recycling for the first time.

[0004] The invention is directed to the bioconversion of bio-based oil to 10-HSA in a cell-free, enzymatic process. The first step of the cascade is the hydrolysis of the bio-based oil with a lipase to gain free fatty acids, especially oleic acid. The second step of the cascade is the hydration of the free oleic acid with an oleate hydratase.

Short description of the Figures

[0005]

Figure 1: Sequence alignment of 14 different OHs from *Rhodococcus erythropulus* (Rre), *Staphylococcus aureus* (Sau), *Lysinibacillus fusiformis* (Lfu), *Macrococcus caseolyticus* (Mca), *Lactobacillus acidophilus* (Lac), *Ochrobactrum anthropi* (Oan), *Bifidobacterium breve* (Bbr), *Streptococcus pyogenes* (Spy), *Elizabethkingia meningoseptica* (Eme), *Myroides odoratus* (Mod), *Cellulophaga algicola* (Cal), *Stenotrophomonas maltophilia* (Sma), *Corynebacterium kroppenstedtii* (Ckr) and *Chryseobacterium gleum* (Cgl).

Figure 2: Phylogenetic tree of the 14 aligned oleate hydratases in figure 1. Created by BLAST with the following settings: Tree method: Fast Minimum Evolution; Max Seq Difference: 0.85; Distance: Grishin (protein).

Figure 3: GC-MS chromatogram after hydration of oleic acid (purity ≈ 80 %; 35.4 mM) with the oleate hydratase from *Stenotrophomonas maltophilia* (final conc. 5 μM enzyme) for 90 min. RT 22.72: C12:0; RT 32.80: palmitic acid; RT 37.26: stearic acid; RT 37.76: oleic acid; RT 38.62: linoleic acid; RT 48.67: 10-hydroxystearic acid (10-HSA).

Figure 4: GC-FID chromatogram after hydration of oleic acid (pure; 720 μM) with the oleate hydratase from *Rhodococcus erythropulus* (final conc. 5 μM enzyme) for 15 min. RT 7.5: oleic acid; RT 15.6: 10-hydroxystearic acid (10-HSA).

Figure 5: Results of activity check of different samples after extraction on TCL plates after scale-up pre-testing of the conversion of oleic acid (OA) to 10-hydroxystearic acid (10-HSA) by the oleate hydrates (OH) from *Stenotrophomonas maltophilia* (ohySm) under different reaction conditions (Oleic acid (OA); 10-hydroxystearic acid (10-HSA); 1.1: 1st conversion; 1.2: 2nd conversion (recycled enzyme); 1.3: filtrate after 2nd conversion).

Figure 6: Resulting pellets of the preparation 1 and 2 of the filtration test.

Figure 7: SDS-PAGE analysis results of filtration test.

Figure 8: Results of activity check of the samples by extraction and TCL of filtration test.

Figure 9: Extraction results of the filter cake and the filtering cloth after direct extraction with EtOAc and spotting on TLC plates.

Figure 10: Documentation of the fermentation process, monitoring all relevant fermentation parameters.

Figure 11: SDS-PAGE corresponding to collected samples in Table. 6. Sample vol. 5 μl. M: page ruler unstained protein ladder (5 μl).

Figure 12: SDS-PAGE corresponding to collected samples in Table. 6. Sample vol. 2.5 μl. M: page ruler unstained protein ladder (5 μl).

Figure 13: TLC plate results after extraction test of samples 7 & 8 with OA and 10-HSA as standards.

Figure 14: 1 ml activity tests on TLC plate from collected samples with OA and 10-HSA as standards.

Figure 15: Activity monitoring after 60 min reaction time from the two 4 L reactions with OA and 10-HSA as standards (TCL plate).

Figure 16: Resulting filter cakes by filtration step with miracloth fabric (4 layers).

Figure 17: Resulting pellet after separation of the product from the recycled enzyme solution.

Figure 18: TLC analysis from the separation approach.

Detailed description of the invention

[0006] Before describing the invention in detail, it is deemed expedient to provide definitions for certain technical terms used throughout the description. Although the present invention will be described with respect to particular embodiments, this description is not to be construed in a limiting sense. Before describing in detail exemplary embodiments of the present invention, definitions important for understanding the present invention are given.

Definitions

[0007] As used in this specification and in the appended claims, the singular forms of "a" and "an" also include the respective plurals unless the context clearly dictates otherwise.

[0008] In the context of the present invention, the terms "about" and "approximately" denote an interval of accuracy that a person skilled in the art will understand to still ensure the technical effect of the feature in question. The term typically indicates a deviation from the indicated numerical value of ±20 %, preferably ±15 %, more preferably ±10 %, and even more preferably ±5 %.

[0009] It is to be understood that the term "comprising" is not limiting. For the purposes of the present invention the term "consisting of" is considered to be a preferred embodiment of the term "comprising of". If hereinafter a group is defined to comprise at least a certain number of embodiments, this is meant to also encompass a group which preferably consists of these embodiments only.

[0010] Furthermore, the terms "first", "second", "third" or "(a)", "(b)", "(c)", "(d)" etc. and the like in the description and in the claims, are used for distinguishing between similar elements and not necessarily for describing a sequential or chronological order. It is to be understood that the terms so used are interchangeable under appropriate circumstances and that the embodiments of the invention described herein are capable of operation in other sequences than described or illustrated herein.

[0011] In case the terms "first", "second", "third" or "(a)", "(b)", "(c)", "(d)" etc. relate to steps of a method or use there is no time or time interval coherence between the steps, i.e. the steps may be carried out simultaneously or there may be time intervals of seconds, minutes, hours, days, weeks, months or even years between such steps, unless otherwise indicated in the application as set forth herein above or below.

[0012] Herein below, various embodiments of the invention are explained in more detail. Wherever, respective alternatives in terms of ingredients in compositions, concentrations or amounts of ingredients, periods of time, the person skilled in the art would immediately understand that individual combinations can be made as long as these are technically possible or if not otherwise explicitly indicated.

[0013] The present invention describes a cell free, enzymatic reaction sequence to convert bio-based mono-, di- or triglycerides (oils) via a free fatty acid (FFA) intermediate (oleic acid) into 10-hydroxystearic acid (10-HSA).

[0014] Generally all animal, plant or microbial (i.e. triglycerides derived from bacteria, yeast, algae or fungi) oils are suitable as feedstocks for the described enzymatic conversion steps.

[0015] Bio-based triglycerides with an oleic acids content above 40% can be used for this process. Examples are castor oil, tall oil or the triglyceride fraction from the oleaginous yeast *Rhodosporidium toruloides.*

[0016] In some embodiments bio-based triglycerides with an oleic acid content above 54% are used as feedstocks for the described process. A specific example is the triglyceride fraction of the oleaginous yeast *Cutaneotrichosporon oleaginosus* or rapeseed oil.

[0017] In further embodiments, oil feedstocks are bio-based oils with an oleic acid content above 75%. Examples are native sunflower oil or high oleic acid sunflower oil variants thereof (see Example 1 and Example 2).

[0018] Subject-matter of the present application is a process for the cell-free enzymatic production of 10-hydroxystearic acid (10-HSA) comprising the following steps:

1) Enzymatic hydrolysis of oil comprising at least 25% oleic acid using lipase to provide free fatty acids comprising oleic acid, and

2) Hydration of the free fatty acids using oleate hydratase (EC 4.2.1.53),
wherein the oleate hydratase is selected from *Stenotrophomonas maltophilia* or derivatives thereof having at least 10% activity when compared with wild-type enzyme under the same conditions or active fragments thereof and/or *Rhodococcus erythropolis* or derivatives thereof having at least 10% activity when compared with wild-type enzyme under the same conditions or active fragments thereof.

3) Separation of 10-HSA in form of a filter cake from the reaction mixture, and

4) Preparation of 10-HSA from the separated filter cake,

and wherein, optionally, the lipase and/or the hydratase used in step 1) and /or step 2) is/are recycled and/or immobilised.

[0019] Optionally, the enzymatic hydrolysis according to step 1 may also be performed with oil comprising at least 30%, 35%, 40%, 45%, 50%, 55%, 60%, 65% or 70% oleic acid.

Optionally, the hydration of the free fatty acids using according to step 2 may also be performed with oleate hydratase from *Stenotrophomonas maltophilia* or derivatives thereof having at least 15%, 20%, 30%, 40%, 50%, 60%, 70%, 80%, 90%, 100%, 110%, 120%, 130%, 140%, 150%, 160%. 170%, 180%, 190% or 200% activity when compared with wild-type enzyme under the same conditions or active fragments thereof and/or *Rhodococcus erythropolis* or derivatives thereof having at least 15%, 20%, 30%, 40%, 50%, 60%, 70%, 80%, 90%, 100%, 110%, 120%, 130%, 140%, 150%, 160%. 170%, 180%, 190% or 200% activity when compared with wild-type enzyme under the same conditions or active fragments thereof.

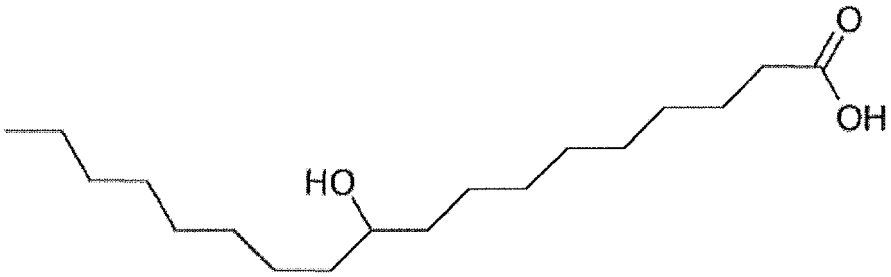
[0020] Each value of content of oleic acid of oil according to step 1 may be combined with each value of activity of oleate hydratase or derivatives thereof according to step 2.

[0021] According to the present invention it is possible to recycle and/or immobilise the lipase and/or the hydratase used in step 1) and /or step 2), and wherein, optionally, the process according to the present invention may be repeated at least once using the recycled and/or immobilised enzyme(s).
The recycled enzyme(s) may be reused at least twice, preferably 2-100 times, 2-50 times, and most preferably 2-10 or 2-5 times, which is a substantial advantage associated with the process of the present invention.

[0022] As used herein, the term "cell free" process refers to a process substantially free of intact cells. One of skill in the art would understand that a certain percentage of the cells after lysing may be intact, e.g., less than 10%, less than 5%, less than 2%, less than 1%, or less than 0.5%. A "cell-free system," as used herein, is an isolated cell-free system containing a cell lysate or extract expressly engineered to include an enzyme or cascade of enzymes that, when acting in a given sequence (e.g., in an enzymatic pathway) and proportion over a determined substrate, results in the generation of a desired product (e.g. a biofuel or other chemical compound, or an intermediate thereto).

[0023] As used herein, the term "enzymatic" process refers to a reaction which is assisted or catalyzed by an enzyme, herein generally classified as a lipase or hydratase and more specifically identified below. Necessary components for the enzymatic reactions include a substrate.

[0024] 10-hydroxystearic acid (10-HSA) (CAS: 638-26-6) has the following formula:



[0025] Both enantiomers may be used according to the present invention.

[0026] The term "fatty acid (FA)", as used herein, refers to any carboxylic acid with an aliphatic tail. Most naturally occurring fatty acids have a chain of an even number of carbon atoms, such as from 4 to 28, and are usually derived from triglycerides or phospholipids. Short-chain fatty acids (SCFA) are fatty acids with aliphatic tails of fewer than 6 carbons (e.g., butyric acid). Medium-chain fatty acids (MCFA) are fatty acids with aliphatic tails of 6-12 carbons, which can form medium-chain triglycerides. Long- chain fatty acids (LCFA) are fatty acids with aliphatic tails 12 to 22 carbons. Very long chain fatty acids (VLCFA) are fatty acids with aliphatic tails longer than 22 carbons. FAs can be either unsaturated or saturated. Unsaturated fatty acids comprise a high percentage of the total fatty acids in plant material consumed by ruminant species. The microbial population that inhabits the rumen transforms dietary unsaturated fatty acids into an array of *trans* fatty acids, conjugated acids, and stearic acid.

[0027] The term "triglyceride", as used herein, refers to an ester derived from glycerol and three fatty acids. There are many different types of triglyceride, with the main division between saturated and unsaturated types. Saturated fats are "saturated" with hydrogen - all available places where hydrogen atoms could be bonded to carbon atoms are occupied. These have a higher melting point and are more likely to be solid at room temperature. Unsaturated fats have double bonds between some of the carbon atoms, reducing the number of places where hydrogen atoms can bond to carbon atoms. These have a lower melting point and are more likely to be liquid at room temperature. Triglycerides are chemically tri esters of fatty acids and glycerol. Triglycerides are formed by combining glycerol with three fatty acid molecules. Alcohols have a hydroxyl (HO-) group. Organic acids have a carboxyl (-COOH) group. The glycerol molecule has three hydroxyl (HO-) groups. Each fatty acid has a carboxyl group (-COOH). In triglycerides, the hydroxyl groups of the glycerol join the carboxyl groups of the fatty acid to form ester bonds:

$$HOCH_2CH(OH)CH_2OH + RCO_2H + R'CO_2H + R"CO_2H \rightarrow RCO_2CH_2CH(O_2CR')CH_2CO_2R" + 3H_2O$$

[0028] The three fatty acids (RCO_2H, $R'CO_2H$, $R"CO_2H$ in the above equation) are usually different, but many kinds of triglycerides are known. The chain lengths of the fatty acids in naturally occurring triglycerides vary, but most contain 16, 18, or 20 carbon atoms. Natural fatty acids found in plants and animals are typically composed of only even numbers of carbon atoms, reflecting the pathway for their biosynthesis from the two-carbon building-block acetyl CoA. Bacteria, however, possess the ability to synthesize odd- and branched-chain fatty acids. As a result, ruminant animal fat contains odd-numbered fatty acids, such as 15, due to the action of bacteria in the rumen. Many fatty acids are unsaturated, some are polyunsaturated (e.g., those derived from linoleic acid). Most natural fats contain a complex mixture of individual triglycerides.

[0029] As used herein, the term "enzymatic hydrolysis" relates to a process in which enzymes facilitate the cleavage of bonds in molecules with the addition of the elements of water.

[0030] Fats and oils are hydrolyzed by moisture to yield glycerol and 3 fatty acids. Chemically fats are esters, so they are liable to hydrolysis. This reaction is catalyzed by a lipase or can occur via non-enzymatic hydrolysis. Partial hydrolysis of triglycerides will yield mono- and diglycerides and free fatty acids. When hydrolysis is carried to completion with water in the presence of an acid catalyst, the mono-, di-, and triglycerides will hydrolyzed to yield glycerol and free fatty acids. Enzyme reactions require milder conditions, less solvent, and give cleaner products attributes of green chemistry. There is increasing interest in the use of lipase enzymes for large-scale reactions. Reaction generally occurs under milder conditions of temperature and pH and there is reduced danger of undesirable side-reactions.

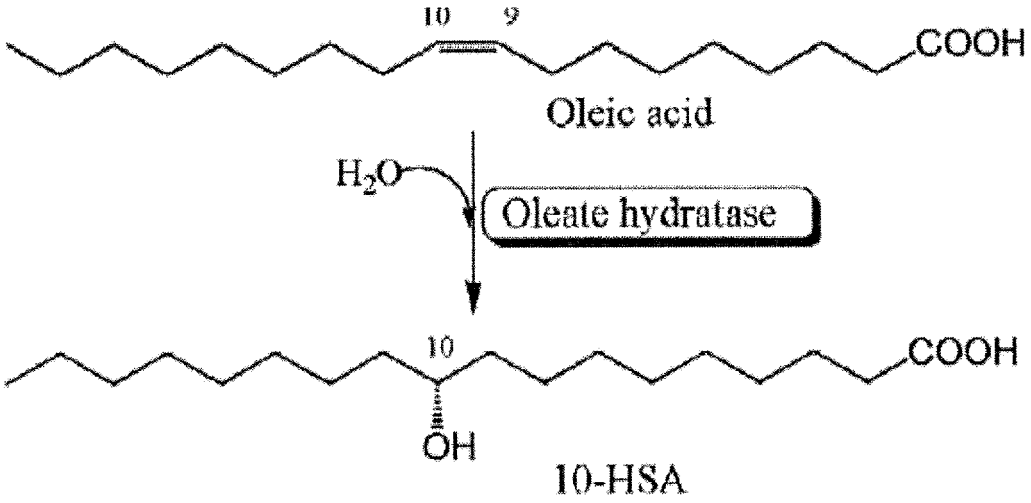
[0031] Oleic acid, as used herein, is a fatty acid that occurs naturally in various animal and vegetable fats and oils. It is an odorless, colorless oil, though commercial samples may be yellowish. In chemical terms, oleic acid is classified as a monounsaturated omega-9 fatty acid, abbreviated with a lipid number of 18:1 *cis*-9. It has the formula $CH_3(CH_2)_7CH=CH(CH_2)_7COOH$. The term "oleic" means related to, or derived from, olive oil which is mostly composed of oleic acid.

The term "triglyceride lipase" as used herein, relates to lipases that hydrolyse ester linkages of triglycerides. These lipases are widely distributed in animals, plants and prokaryotes. This family was also called class 3 lipases as they are only distantly related to other lipase families. In particular, the triglyceride lipase EC class 3.1.1.3 ("triacylglycerol lipase"),

as used herein, relates to the pancreatic enzyme that acts only on an ester-water interface, wherein the outer ester links are preferentially hydrolysed. The triacylglycerol lipase (EC 3.1.1.3, *lipase*) catalyses the following chemical reaction : triacylglycerol + H_2O diacylglycerol + a carboxylate.

[0032] As used herein, the term "hydration" relates to a chemical process that introduces a hydroxyl group (-OH) into an organic compound. Hydratases are a group of lyases that catalyze hydration and dehydration of a substrate. Even though many hydratases are known, yet there are only few known oleate hydratases [6][6][6]. Oleate hydratases belong to the group of fatty acid hydratases and convert oleic acid into (R)-10-hydroxystearic acid (EC 4.2.1.53).

[0033] Hydratases (EC 4.2.1.53), as used herein, catalyze the regio-specific, irreversible addition of a hydrogen atom and a hydroxy group from water to the carbon-carbon cis-double bond of unsaturated fatty acids at the C9 and C10 positions, respectively, to make 10-hydroxy fatty acids (Joo et al., 2012a). Oleate hydratases convert oleic acid to 10-hydroxystearic acid as shown in Scheme 1:



Scheme 1: Catalytic mode of hydratase

Oleate hydratase homologies:

[0034] As used herein, "oleate hydratases" have sequence identities of oleate hydratase enzymes from different taxonomic origins are displayed in Figure 1 and 2. With any enzyme of this family the hydration of oleic acid can be carried out with different degrees of efficiency.

[0035] In some embodiments, reactions are carried out with the native oleate hydratases or respective mutants thereof (with a sequence identity of more than 80% and functional for the described application) from *Stenotrophomonas maltophilia* (Sma), *Elizabethkingia meningoseptica* (ohyA), *Macrococcus caseolyticus* (Mca), *Bifidobacterium breve* (Bbr), *Corynebacterium kroppenstedtii* (Ckr), *Ochrobactrum anthropi* (Oan), *Myroides odoratus* (Mod), *Staphylococcus aureus* (Sau), *Chryseobacterium gleum* (Cgl), *Cellulophaga algicola* (Cal), *Rhodococcus erythropulus* (Rre) and *Lactobacillus acidophilus* (Lac).

[0036] In further embodiments, reactions are carried out with the oleate hydratases from *Stenotrophomonas maltophilia* (Sma), *Elizabethkingia meningoseptica* (ohyA) and *Rhodococcus erythropulus* (Rre).

[0037] In additional embodiments, the reaction is carried out with the oleae hydratase from *Stenotrophomonas maltophilia* (Sma, gene *Smlt2093*) published by Joo et al. [7].

[0038] In another embodiment of the present application, the specificity of the hydration of oleic acid with the oleate hydratase from *Stenotrophomonas maltophilia* can be detected using GC-MS chromatography (Figure 3; RT 22.72: C12:0; RT 32.80: palmitic acid; RT 37.26: stearic acid; RT 37.76: oleic acid; RT 38.62: linoleic acid; RT 48.67: 10-hydroxystearic acid (10-HAS)). The direct transesterification of the hydration reaction products was performed according to a modified protocol of Griffiths et al. [8] with the following modifications: replacement of the C17-TAG by a C12-TAG, replacement of BF3 methanol by a HCL-methanol solution, and the C19-ME was omitted. Subsequently, the resulting fatty acid methyl ester (FAME) extract was injected into a Thermo Scientific™ TRACE™ Ultra Gas Chromatograph coupled to a Thermo DSQ™ II mass spectrometer and the Triplus™ Autosampler injector. Column: Stabilwax® fused silica capillary (30 m x 0,25mm, film thickness 0.25 μm). (Program: initial column temperature 50°C, increasing (4 °C/min) up to a final temperature of 250°C. Carrier gas: hydrogen, flow rate 3,5mL/min.) Peaks were identified by comparison to a marine oil standard (Restek) or by specific molecular masses detected. The GC-MS chromatogram shows a high

conversion level from OA to 10-HSA by the oleate hydratase from S. *maltophilia.* This high conversion level is achieved, although the converted substrate was containing contaminants, like palmitic acid, that were reported to decrease the conversion efficiency of the oleate hydratase reaction. [5a] These results demonstrate, that an efficient hydration reaction can be carried out with the oleate hydratase from *S. maltophilia,* even if the utilized substrate in not only pure OA.

[0039] In another embodiment of the present application, the specificity of the hydration of oleic acid with the oleate hydratase from Rhodococcus erythropulus can be detected using GC-FID chromatography (Figure 4, RT 7.5: oleic acid; RT 15.6: 10-hydroxystearic acid (10-HSA)).

[0040] The preparation of the extracted lipid fractions for the GC measurements was performed according to Volkov et al. [9] Extracts were analyzed with the Shimadzu™ GC-2025 system equipped with a flame ionization detector. Column: Zebron ZB-WAX (30 m x 0,32 mm, film thickness 0,25 μm) Phenomenex. Carrier gas: hydrogen (3.00 ml/min). Program: initial column temperature 150 °C for 1 min; increasing 5°C/min to 240°C, hold for 6 min. Peaks were identified by comparison to the respective standards or previous GC-MS results. The results depicted in Figure 4 demonstrate the efficient conversion of pure OA to 10-HSA by the oleate hydratase from *R. erythropolis.* Approximately 95 % of the applied substrate is converted within 15 min under the given conditions.

[0041] A further embodiment of the present invention is the process according to the preceding embodiments, wherein said oil in step 1 is selected from the group comprising renewable/regrowing feedstocks (of bio-based oils).

[0042] The term(s) "renewable" or "regrowing", as used herein, means that something is capable of being renewed or is a substance of economic value that can be replaced or replenished in the same or less amount of time as it takes to draw the supply down. Some renewable resources are considered renewable even though some time or effort must go into their renewal.

[0043] As used herein, the term "feedstocks" refers to raw materials (input) fed into a process for conversion into something different (output). For example, crude oil is a feedstock raw material providing finished products in the fuel, plastic, industrial chemical and pharmaceutical industries. The term "raw material" is used to denote material is in an unprocessed or minimally processed state.

[0044] In another embodiment the present invention relates to the process as defined in any one of the preceding embodiments, wherein said feedstocks are selected from the group comprising animals, plants and microorganisms.

[0045] In another embodiment the present application relates to the process as defined in any one of the preceding embodiments, wherein said oil comprises mono-, di- or triglycerides.

[0046] In another embodiment the present application relates to the process as defined in any one of the preceding embodiments, wherein said oil comprises triglycerids with an oleic acid content ≥ 40 %.

[0047] In another embodiment the present application relates to the process as defined in any one of the preceding embodiments, wherein said oil comprises triglycerids with an oleic acid content ≥ 50 %.

[0048] In another embodiment the present application relates to the process as defined in any one of the preceding embodiments, wherein said oil comprises triglycerids with an oleic acid content ≥ 70%.

[0049] In another embodiment the present application relates to the process as defined in any one of the preceding embodiments, wherein said oil is a plant oil selected from the group comprising vegetable oil, tree borne oil, olive oil, mustard oil, linseed oil, canola oil, coconut oil, coriander oil, corn oil, cottonseed oil, hazelnut oil, olive oil, neem oil, palm oil, peanut oil, rapeseed oil, rice bran oil, safflower oil, soybean oil, sunflower seed oil, and mixtures thereof.

[0050] In another embodiment the present application relates to the process as defined in any one of the preceding embodiments, wherein said oil is an animal oil selected from the group comprising tall, fish and crustacean oils.

[0051] In another embodiment the present application relates to the process as defined in any one of the preceding embodiments, wherein said oil is a microbial oil.

[0052] In another embodiment the present application relates to the process as defined in any one of the preceding embodiments, wherein said oil is derived from bacteria, yeast, algae and/or fungi.

[0053] In another embodiment the present application relates to the process as defined in any one of the preceding embodiments, wherein said oil is a microbial oil from oleganious yeast, algae and molds. Examples are oils from *Nannochloropsis salina* (algae), *Rhodospirillum tourolides (bacteria), Trichosporon oleganosus (fungus), Yarrowia lipolytica (yeast).*

[0054] In another embodiment the present application relates to the process as defined in any one of the preceding embodiments, wherein said lipase is selected from the group comprising mono-, di- or triglyceride lipase.

[0055] In another embodiment the present application relates to the process as defined in any one of the preceding embodiments, wherein said lipase is a lipase selected from the EC class of hydrolases (EC class 3), preferably from the EC class of esterase enzymes acting on ester bonds (EC class 3.1), more preferably from the class of carboxylic-ester hydrolases (EC class 3.1.1).

[0056] In another embodiment the present application relates to the process as defined in any one of the preceding embodiments, wherein said lipase is a lipase selected from the EC class of triglyceride lipases (EC class 3.1.1.3).

[0057] In another embodiment the present application relates to the process as defined in any one of the preceding embodiments, wherein said lipase is a triglyceride lipase selected from the group comprising *Candida rugosa* lipase, or

lipase from porcine pancreas, lipase from *Rhizopus oryzae or* lipase from Pseudomonas sp.

[0058] In another embodiment the present application relates to the process as defined in any one of the preceding embodiments, wherein said enzymatic hydrolysis using lipase is carried out in the presence of a catalyst.

[0059] As used herein, the term "catalyst" refers to a substance that speeds up a chemical reaction, but is not consumed by the reaction; hence a catalyst can be recovered chemically unchanged at the end of the reaction it has been used to speed up, or catalyze.

[0060] In another embodiment the present application relates to the process as defined in any one of the preceding embodiments, wherein said catalyst is selected from the group comprising Tween®, Tween-20® or ethanol.

[0061] In another embodiment the present application relates to the process as defined in any one of the preceding embodiments, wherein said enzymatic hydrolysis is carried out in an aqueous system.

[0062] As used herein, the term "aqueous system" comprises an aqueous solution that is any solution in which water (H_2O) is the solvent.

[0063] In another embodiment the present application relates to the process as defined in any one of the preceding embodiments, wherein said aqueous system comprises at least one buffer and/or at least one solvent, mixtures of solvents and/or mixtures of buffer(s) and solvent(s).

[0064] In another embodiment the present application relates to the process as defined in any one of the preceding embodiments, wherein said buffer is selected from Tris-HCl buffer, phosphate-citrate-buffer, 3-(N-morpholino)propanesulfonic acid (MOPS) buffer.

[0065] In another embodiment the present application relates to the process as defined in any one of the preceding embodiments, wherein the concentration of said buffer is in the range of 10 mM to 100 mM, preferably 10 mM to 50 mM, more preferably 15 mM to 40 mM, most preferably 20 mM to 30 mM.

[0066] In another embodiment the present application relates to the process as defined in any one of the preceding embodiments, wherein the concentration of said buffer is 20 mM ± 5 %.

[0067] In another embodiment the present application relates to the process as defined in any one of the preceding embodiments, wherein the *Candida rugosa* lipase is used in 20 mM Tris-HCl buffer with a pH value of 7.2.

[0068] In another embodiment the present application relates to the process as defined in any one of the preceding embodiments, wherein said enzymatic hydrolysis is carried out at a temperature ranging from 10°C to 60 °C.

[0069] In another embodiment the present application relates to the process as defined in any one of the preceding embodiments, wherein said enzymatic hydrolysis is carried out at a temperature ranging from 20°C to 50 °C.

[0070] In another embodiment the present application relates to the process as defined in any one of the preceding embodiments, wherein said enzymatic hydrolysis is carried out at a temperature ranging from 30°C to 40 °C.

[0071] In another embodiment the present application relates to the process as defined in any one of the preceding embodiments, wherein said enzymatic hydrolysis is carried out at a temperature of 37°C ± 5 %.

[0072] In another embodiment the present application relates to the process as defined in any one of the preceding embodiments, wherein said enzymatic hydrolysis is carried out for a period of 15 to 300 minutes, preferably 30 to 180 minutes, more preferably 60 - 90 min.

[0073] In another embodiment the present application relates to the process as defined in any one of the preceding embodiments, wherein after the enzymatic hydrolysis, glycerol is separated from the free fatty acids in a washing step using water/buffer and/or by extracting the free fatty acids from the reaction mixture using at least one organic solvent and/or by phase separation.

[0074] As used herein, the term "extraction" relates to a way to separate a desired substance when it is mixed with others. The mixture is brought into contact with a solvent in which the substance of interest is soluble, but the other substances present are insoluble. Extractions use two immiscible phases (these are phases that do not mix, like oil and water) to separate the substance from one phase into the other.

[0075] In one embodiment the present application relates to the process as defined in any one of the preceding embodiments, wherein said enzymatic hydrolysis of oil using lipase (step 1) and the hydration using oleate hydratase (step 2) can be carried out sequentially or simultaneously (see Scheme 2, Process option I or II).

[0076] In one embodiment the present application relates to the process as defined in any one of the preceding embodiments, wherein in a first step the enzymatic hydrolysis of oil using lipase is performed. Following this reaction, a washing/purification step is carried out, wherein the free fatty acids (e.g. oleic acid) are separated from the residual reaction mixture.

[0077] As used herein, the term "purification" in chemistry relates to a separation of a substance into its components and refers to the process of removing impurities.

[0078] As used herein, the term "separation" relates to a process to achieve any phenomenon that converts a mixture of chemical substance into two or more distinct product mixtures, which may be referred to as mixture, at least one of which is enriched in one or more of the mixture's constituents. In some cases, a separation may fully divide the mixture into its pure constituents. Separations differ in chemical properties or physical properties such as size, shape, mass, density, or chemical affinity, between the constituents of a mixture. They are often classified according to the particular

differences they use to achieve separation. Usually there is only physical movement and no substantial chemical modification. If no single difference can be used to accomplish a desired separation, multiple operations will often be performed in combination to achieve the desired end.

[0079] In another embodiment the present application relates to the process as defined in any one of the preceding embodiments, wherein in a first step (step 1) the enzymatic hydrolysis of oil using lipase is performed resulting in a mixture of buffer, enzyme, glycerin,oleic acid or a mixture of free fatty acids (FFAs) comprising oleic acid, wherein the main component of the free fatty acids is oleic acid. Subsequently, water and glycerin are separated from the free fatty acids (FFAs) which are dissolved in at least one organic solvent. The end product, the FFAs, is obtained by removal of the organic solvent(s), wherein said solvent can be used for another wash/purification step.

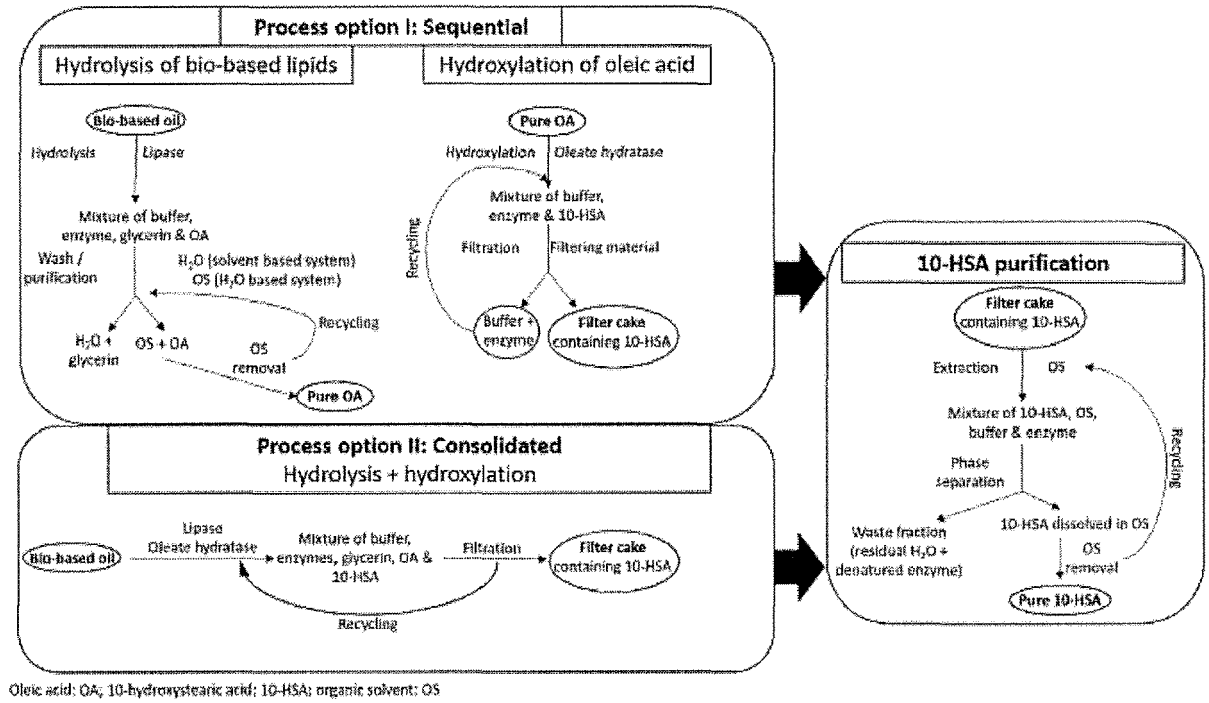
[0080] In another embodiment the present application relates to the process as defined in any one of the preceding embodiments, wherein in a second step (step 2) the enzymatic hydration of oleic acid using oleate hydratase is performed resulting in a mixture of buffer, enzyme, residual amounts of oleic acid, for example, ≤ 10 %, ≤ 7.5, ≤ 5 %, or even below, and 10-HSA. Subsequently, the precipitated 10-HSA is separated as a filter cake containing 10-HSA and the remaining ingredients of the reaction mixture comprising buffer and enzyme are recycled for a new reaction (see scheme 2, Process option I).

[0081] The term "filter cake", as used herein, is formed by the substances that are retained on a filter. The filter cake grows in the course of filtration, becomes "thicker" as particulate matter is being retained. With increasing layer thickness the flow resistance of the filter cake increases.

[0082] After a certain time of use the filter cake has to be removed from the filter, e.g. by backflushing. If this is not accomplished, the filtration is disrupted because the viscosity of the filter cake gets too high, thus too little of the mixture to be filtered can pass through the filter cake and the filter plugs. The specifications of the filter cake dictate the filtration method of choice.

[0083] In another embodiment the present application relates to the process as defined in any one of the preceding embodiments, wherein the enzymatic hydrolysis of oil using lipase and the hydration using oleate hydratase is performed simultaneously. Following this reaction, the product, 10-HSA, is separated from the reaction mixture (using filtration) and the remaining ingredients of the reaction mixture are recycled for a new reaction.

[0084] In another embodiment the present application relates to the process as defined in any one of the preceding embodiments, wherein said enzymatic hydrolysis of oil using lipase (step 1) and the hydration using oleate hydratase (step 2) is performed simultaneously, and subsequently, the precipitated product, 10-HSA, is separated from the reaction mixture comprising buffer, enzymes, glycerin and oleic acid using filtration. The precipitated 10-HSA is thereby separated as a filter cake and the remaining ingredients of the reaction mixture are recycled for a new reaction (see scheme 2, Process option II).



Oleic acid: OA; 10-hydroxystearic acid: 10-HSA; organic solvent: OS

Scheme 2: Different options for the process of the present invention

[0085] In another embodiment the present application relates to the process as defined in any one of the preceding embodiments, wherein the purification of 10-HSA comprises the following steps: a) an extraction with at least one organic solvent, and

b) a phase separation.

[0086] In another embodiment the present application relates to the process as defined in any one of the preceding embodiments, wherein the purification of 10-HSA comprises an extraction with at least one organic solvent, wherein a mixture of 10-HSA, organic solvent, buffer, and enzymes are extracted from the filter cake containing 10-HSA. Subsequently, 10-HSA which is dissolved in at least one organic solvent is separated by phase separation from a waste fraction comprising residual water and denatured enzymes. The end product 10-HSA is obtained by removing the organic solvent(s), wherein said solvent(s) can be reused for the extraction from the filter cake (see scheme 2, box on the right).

[0087] In another embodiment the present application relates to the process as defined in any one of the preceding embodiments, wherein after the enzymatic hydrolysis (step 1) said free fatty acids are optionally separated from the reaction mixture by at least one washing/purification step.

[0088] In another embodiment the present application relates to the process as defined in any one of the preceding embodiments, wherein after the enzymatic hydrolysis (step 1), said free fatty acids are separated from glycerol in at least one washing/purification step by extraction or phase separation.

[0089] In another embodiment the present application relates to the process as defined in any one of the preceding embodiments, wherein after the enzymatic hydrolysis (step 1) said free fatty acids are optionally separated from the reaction mixture by at least one washing/purification step using at least one organic solvent and/or an aqueous system.

[0090] In another embodiment the present application relates to the process as defined in any one of the preceding embodiments, wherein said organic solvent for the washing/purification of the free fatty acids is selected from the group comprising ethyl acetate, hexane, toluene, methyl isobutyl ketone (MIBK), methanol, ether.

[0091] In another embodiment the present application relates to the process as defined in any one of the preceding embodiments, wherein said organic solvent is ethyl acetate.

[0092] In another embodiment the present application relates to the process as defined in any one of the preceding embodiments, wherein said organic solvent for the washing/purification of the free fatty acids is used in an amount which corresponds to the solubility of the free fatty acids or 10-HSA in the solvent, respectively.

[0093] In another embodiment the present application relates to the process as defined in any one of the preceding embodiments, wherein said hydration using oleate-hydratase (step 2) is carried out in an aqueous system.

[0094] In another embodiment the present application relates to the process as defined in any one of the preceding embodiments, wherein said aqueous system for the hydration comprises buffer, free fatty acids as substrate, oleate hydratase and/or emulsifier.

[0095] In another embodiment the present application relates to the process as defined in any one of the preceding embodiments, wherein said emulsifier is selected from the group comprising Tween®, Tween-20® or ethanol.

[0096] In another embodiment the present application relates to the process as defined in any one of the preceding embodiments, wherein said hydration is carried out in a buffer selected from the group comprising Tris-HCl buffer, phosphate-citrate-buffer, 3-(N-morpholino)propanesulfonic acid (MOPS) buffer, 2-(N-morpholino)ethanesulfonic acid (MES) buffer, piperazine-N,N'-bis(2-ethanesulfonic acid) (PIPES); 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES); Sørensen's phosphate buffer (Stock solutions: A 0.2 M NaH_2PO_4, B 0.2 M Na_2HPO_4).

[0097] In another embodiment the present application relates to the process as defined in any one of the preceding embodiments, wherein the hydration using oleate hydratase from *ohySm* is carried out in 50 mM phosphate-citrate-buffer with a pH value of 6.5.

[0098] In another embodiment the present application relates to the process as defined in any one of the preceding embodiments, wherein said hydration is carried out at a temperature ranging from 10°C to 50°C, preferably 20 -40 °C, more preferably 25 - 35 %.

[0099] In another embodiment the present application relates to the process as defined in any one of the preceding embodiments, wherein said hydration results in at least 25, 50, 75, 100 %, or more conversion of said fatty acids (comprising oleic acid) to 10 -HSA.

[0100] In another embodiment the present application relates to the process as defined in any one of the preceding embodiments, wherein said hydration is carried out for a period of 10 to 300 minutes, preferably 30 to 180 minutes, more preferably 60 to 90 minutes.

[0101] In another embodiment the present application relates to the process as defined in any one of the preceding embodiments, wherein the hydration is carried out under constant mixing, wherein the speed is in the range of 250 und 750 rpm.

[0102] In another embodiment the present application relates to the process as defined in any one of the preceding embodiments, wherein the product of the hydration (step 2), 10-HSA may form flocks that can be separated from the

mixture by filtration, precipitation, sedimentation or gravimetric solid-liquid-separation.

[0103] The term flocculation, as used herein, is a process wherein colloids come out of suspension in the form of flock or flake, either spontaneously or due to the addition of a clarifying agent. The action differs from precipitation in that, prior to flocculation, colloids are merely suspended in a liquid and not actually dissolved in a solution. In the flocculated system, there is no formation of a cake, since all the flocks are in the suspension.

[0104] Optionally, it is possible to add a flocculation agent.

[0105] In another embodiment the present application relates to the process as defined in any one of the preceding embodiments, wherein the size of the flocks is further dependent on the pH value of the reaction mixture.

[0106] In another embodiment the present application relates to the process as defined in any one of the preceding embodiments, wherein said flocks preferably tend to form in an alkaline pH range.

In another embodiment the present application relates to the process as defined in any one of the preceding embodiments, wherein said pH value is in a range from 5-10.

[0107] In another embodiment the present application relates to the process as defined in any one of the preceding embodiments, wherein said pH value is in a range from 5-8.

[0108] In another embodiment the present application relates to the process as defined in any one of the preceding embodiments, wherein an emulsion breaker is used additionally to improve the formation of flocks.

[0109] In another embodiment the present application relates to the process as defined in any one of the preceding embodiments, wherein after the hydration (step 2) the precipitated 10-HSA is separated from the reaction mixture as filter cake.

[0110] In another embodiment the present application relates to the process as defined in any one of the preceding embodiments, wherein said filter cake comprises 10-HAS.

[0111] In another embodiment the present application relates to the process as defined in any one of the preceding embodiments, wherein after the hydration (step 2) the precipitated 10-HSA is separated from the reaction mixture as filter cake using filtration and/or centrifugation and/or using chromatographic methods.

[0112] In another embodiment the present application relates to the process as defined in any one of the preceding embodiments, wherein after the hydration product, 10-HSA, is optionally separated from the reaction mixture as filter cake by at least one filtration step using a filter.

[0113] In another embodiment the present application relates to the process as defined in any one of the preceding embodiments, wherein after the hydration product, 10-HSA, is optionally separated from the reaction mixture using chromatographic methods, for example, by hydrophobic adsorber packed in a column.

[0114] In another embodiment the present application relates to the process as defined in any one of the preceding embodiments, wherein after the hydration (step 2) the precipitated 10-HSA is separated from the reaction mixture as filter cake by at least one filtration step using a plate filter press.

[0115] In another embodiment the present application relates to the process as defined in any one of the preceding embodiments, wherein said filter is selected from the group comprising a deep bed filter, fabric with pores of a size up to 30 μm, miracloth (rayon-polyester + acrylic binder), cellulose filter with pores of a size up to 30 μm.

[0116] In another embodiment the present application relates to the process as defined in any one of the preceding embodiments, wherein after the hydration (step 2), 10-HSA is prepared from the filter cake using extraction with at least one organic solvent and/or using phase generation.

[0117] In another embodiment the present application relates to the process as defined in any one of the preceding embodiments, wherein after the hydration (step 2), 10-HSA is prepared from the filter cake using extraction with at least one organic solvent, wherein the 10-HSA is dissolved in the at least one organic solvent and afterwards the organic phase is separated from the denatured protein and the residual water.

[0118] In another embodiment the present application relates to the process as defined in any one of the preceding embodiments, wherein after the hydration (step 2), 10-HSA is prepared from the filter cake using extraction with at least one organic solvent, wherein water is added prior extraction in order to improve the formation of delimited phases.

[0119] In another embodiment the present application relates to the process as defined in any one of the preceding embodiments, wherein after the hydration (step 2), 10-HSA is prepared from the filter cake using extraction with at least one organic solvent, wherein said organic solvent is selected from the group comprising ethyl acetate, hexane, methanol, ether, methyl-isobutyl-ketone, toluene.

[0120] In another embodiment the present application relates to the process as defined in any one of the preceding embodiments, wherein the concentration of said organic solvent is between 80% w/v to 100% w/v.

[0121] In another embodiment the present application relates to the process as defined in any one of the preceding embodiments, wherein the concentration of said organic solvent is $\leq$ 90% w/v.

[0122] In another embodiment the present application relates to the process as defined in any one of the preceding embodiments, wherein after the extraction of 10-HSA from the filter cake, an isolation/ purification of 10-HSA is performed (step 4) using phase separation.

[0123] In another embodiment the present application relates to the process as defined in any one of the preceding

embodiments, wherein said purification of 10-HSA from the filter cake (step 4) is performed by phase separation, wherein the dissolved 10-HSA is separated from the residual fraction/reaction mixture comprising water and enzymes.

[0124] In another embodiment the present application relates to the process as defined in any one of the preceding embodiments, wherein said purification of 10-HSA from the filter cake (step 4) is performed by phase separation, wherein 10-HSA is dissolved in at least one organic solvent. The extraction of 10-HSA with at least one organic solvent, as used herein, facilitates a separation of the product from other contaminants (e.g. catalysts, buffer salts etc.).

[0125] In another embodiment the present application relates to the process as defined in any one of the preceding embodiments, wherein said organic solvent for the extraction of 10-HSA is selected from the group comprising ethyl acetate, hexane, methanol, ether, methyl-isobutyl-ketone, toluene.

[0126] In another embodiment the present application relates to the process as defined in any one of the preceding embodiments, wherein the concentration of said organic solvent is between 80% w/v to 100% w/v.

[0127] In another embodiment the present application relates to the process as defined in any one of the preceding embodiments, wherein the concentration of said organic solvent is $\leq$ 90% w/v.

[0128] In another embodiment the present application relates to the process as defined in any one of the preceding embodiments, wherein the organic solvent is removed by evaporation, distilling, gassing with nitrogen and/or phase separation.

[0129] In another embodiment the present application relates to a composition or product comprising 10-HSA obtainable by the process according to any of the preceding embodiments. In one embodiment, said composition or product is selected from specialty oleochemicals, chemical performance additives, cosmetics, cosmetic additives, in particular lubricants. In one embodiment, said composition or product comprising 10-HAS obtainable by the process according to any of the preceding embodiments is used as a specialty oleochemical, chemical performance additive, cosmetic, or cosmetic additive, in particular as a lubricant.

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[0130]

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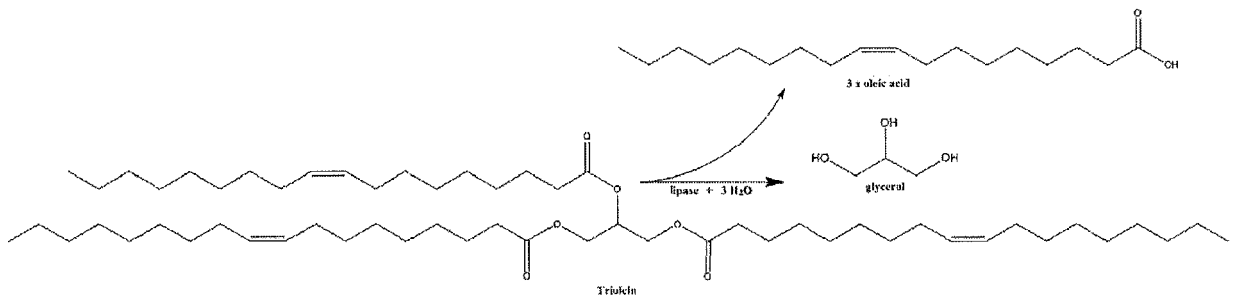
Examples/Experiments:

1.) Hydrolysis of high oleic sunflower oil (HOSO), as an example for a bio-based oil

[0131] The conventional lipase based hydrolysis of bio-based oil is known to those skilled in the art (Enzymatic process for fat and oil hydrolysis, WO 2013114178 A1). More specifically, the hydrolysis of the HOSO, consisting of over 90% triolein, is carried out by a lipase (EC 3.1.1.3). An appropriate catalyst is chosen and the reaction is carried out under its corresponding conditions. After the lipolytic cleavage of the triolein the glycerol is separated from the FFAs by either an additional washing step with water / buffer or by extracting the FFAs from the reaction mixture with an organic solvent. Alternative extraction methods for free fatty acids, such as distillation are known to those skilled in the art.



Scheme 3: Reaction scheme of lipase catalyzed hydrolysis of triolein

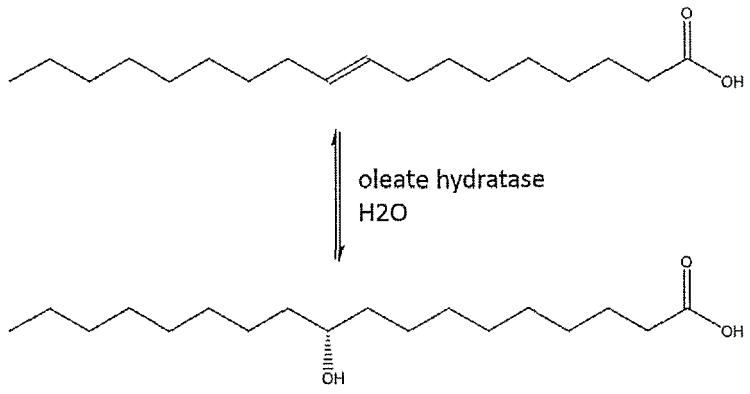
[0132] Commercially available lipase from *Candida rugosa* is used to hydrolyze HOSO under mild conditions in an aqueous reaction system (10 mg/ml lipase; 20 mM HOSO; 20 mM Tris-HCl buffer pH 7,2). After the reaction, the produced glycerol is removed by a washing step with water and the FFAs are extracted from the reaction mixture by the addition of ethyl acetate and a subsequent separation of the organic and aqueous phase. The organic solvent is removed by evaporation or gassing with nitrogen. The obtained FFAs are used for following experiments.

2.) Oleate hydratase catalyzed hydration of oleic acid

[0133] The second step in the process from bio-based oils to 10-HSA is the addition of a hydroxyl group to the carbon chain of the unsaturated FA, which is carried out by an oleate hydratase (EC 4.2.2.53). An appropriate catalyst is chosen and the reaction is carried out under its corresponding conditions. After the addition of the hydroxyl group to the fatty acid chain the product is separated from the reaction mixture by one of the following methods.

[0134] The first method is the direct exposure of the reaction mixture to an organic solvent, e.g. ethyl acetate, in an adequate volume. The produced aqueous and organic phases are subsequently separated and the organic solvent is removed by evaporation, or recovered by distilling. After the removal of the organic solvent, the 10-HSA appears as a crystalline, white wax.

[0135] In an adjacent step the product is separated by a filtering step before the addition of an organic solvent. The additional filtering step reduces the amount of organic solvent for the extraction and allows a partial recovery of the enzyme solution applied for the hydration reaction. The concentration of the product from the reaction mixture can be performed by different filtering methods. The first option is the usage of a suitable deep bed filter. The second option is the usage of a suitable fabric with small pores of a size up to 30 μm. These filtering methods reduce the volume to be extracted by the organic solvent by approximately 90 %. The resulting filter cake is then extracted as described in the first method. The resulting filtrate was shown to exhibit enough recycled, active enzyme to perform multiple hydration steps.



Scheme 4: Reaction scheme of the hydration of oleic acid to 10-hydroxystearic acid by an oleate hydratase.

3.) Hydration of FFAs using oleate hydratase from *Stenotrophomonas maltophilia (strain K279a)*

[0136] The gene *Smlt2093,* coding for a putative myosin-cross-reactive antigen, from *Stenotrophomonas maltophilia (strain K279a),* was taken as a template for a codon-optimized gene-synthesis (life-technologies), for an E. coli host strain. The obtained synthetic gene was sub-cloned in a pET28a expression plasmid and transformed into chemically competent E. coli BL21DE3 cells. The oleate hydratase is heterologously expressed in E. coli BL21DE3 cells, grown in Laure Broth (LB) medium. After an appropriate time of protein expression, cells are harvested from the culture and disrupted by high pressure homogenization. The cell-debris is separated from the oleate hydratase containing liquid phase and discarded.

[0137] The resulting lysate is then used for the hydration of the FFAs-fraction from step 1). The reaction is carried out, as described by Joo et al.[7], in an aqueous system at low temperatures under constant mixing. After an appropriate reaction time the product (10-HSA) can be separated from the reaction mixture by one of the methods described above.

4.) Hydration of FFAs using oleate hydratase from *Rhodococcus erythropolis CCM2595*

[0138] The myosin-cross-reactive antigen coding gene *O5Y_00450* from *Rhodococcus erythropolis CCM2595,* was taken as a template for a codon-optimized gene-synthesis (life-technologies), for an E. coli host strain. The obtained synthetic gene was sub-cloned in a pET28a expression plasmid and transformed into chemically competent E. coli BL21DE3 cells. The native oleate hydratase or respective mutants thereof are expressed in E. coli BL21DE3 cells, grown in Laure Broth (LB) medium. After an appropriate time of protein expression, cells are harvested from the culture and disrupted by high pressure homogenization. The cell-debris is separated from the oleate hydratase containing liquid phase and discarded.

[0139] The resulting lysate is then used for the hydration of the FFAs-fraction from step 1). The reaction is carried out in an aqueous system at low temperatures under constant mixing. After an appropriate reaction time the product (10-HSA) can be separated from the reaction mixture by one of the methods described above.

5.) Scale-up of the 10-HSA production from oleic acid by the oleate hydrates from *Stenotrophomonas maltophilia*

[0140] 5. 1.) Pre-tests for the conversion of oleic acid (OA) to 10-hydroxystearic acid (10-HSA) by the oleate hydrates (OH) from *Stenotrophomonas maltophilia* (ohySm):

Table 1:

Conversion of oleic acid (OA) to 10-hydroxystearic acid (10-HSA) by the oleate hydrates (OH) from *Stenotrophomonas maltophilia* (ohySm) under different reaction conditions (variation of culture conditions and reaction time)			
Sample	Reaction type	Specification	reaction time [h]
1	flat flask (300ml)	1st run	2
1.1		2nd run (recycle)	1.5
2	baffled flask (300ml)	1st run	1.5
2.1		2nd run (recycle)	1.5
3	stirrer 500 rpm	1st run	1.5
3.1		2nd run (recycle)	1.5
4	stirrer 750 rpm	1st run	2.5
5		1st run	1.5

In order to check the activity of the different samples, said samples are extracted and fractions of each sample are spotted on TLC plates (see Figure 5).

The results of the activity tests, in different reaction vessels and by different mixing methods, showed a high conversion level of OA to 10-HSA by oleate hydratase from *ohySm* (Figure 5). No loss in activity is detected after the recycling of the enzyme solution.

5.2.) New pre-tests for filtering the final reaction mixture with miracloth (rayon-polyester + acrylic binder) filtering fabric. Beaker + magnetic stirrer at 250 rpm standard conditions in duplicate. Filtering after first 90 min worked very well.

[0141] The resulting pellet size of the two preparation were documented (Figure 6)

[0142] The following samples were collected:

Table 2:

Overview of sample set for the filtration test	
sample name	description
1	sample after 1st reaction A
2	sample after 1st reaction B
3	sample after 1st filtration A
4	sample after 1st filtration B
5	sample after 2nd reaction A
6	sample after 2nd filtration A
7	sample after 2nd filtration B
8	sample after 3rd reaction A
M	page-ruler unstained marker
OA	oleic acid
10-HSA	10-hydroxystearic acid

Table 3:

Results of recovery rate of conversion of oleic acid (OA) to 10-hydroxystearic acid (10-HSA) with oleate hydratase from *S. maltophilia* after a first and second reaction (50 mM Phosphate-Citrate-Buffer, pH 6.5, at 35°C). Preparation 1 and 2 have been tested independently under the same conditions.

	reaction 1		reaction 2 (recycle)	
	enzyme solution recovery [%]	filter cake [g]	enzyme solution recovery [%]	filter cake [g]
preparation 1	80	6.3	90	0.38
preparation 2	86	6.8	93	0.66

[0143] After the 2nd reaction almost no product is filtered out by the miracloth anymore, suggesting that the enzyme got inactivated (either during the process or by the filtering with the miracloth) or got adsorbed by the filtering material. To eliminate the possibility of a binding of the enzyme to the filtering material, an SDS-PAGE with samples collected from all fractions was prepared (Figure 7).

[0144] The SDS-PAGE showed no loss of protein in all fractions. These results eliminate the option of potential interactions between the protein and the filtering cloth.

[0145] The activity tests of the different samples by extraction and TLC monitored on TLC plates (Figure 8) show a high conversion rate of OA to 10-HSA by the oleate hydratase from *Stenotrophomonas maltophilia* and an excellent filtering performance for the cloth. The TLC plates also show a high conversion rate after the 1st recycling step. Although the 10-HSA couldn't be found as big aggregates (unlike after the 1st reaction), it got adsorbed by the filtering material, because the product couldn't be identified in the fraction after the filtration step. Lane 8 shows the extraction result after the 2nd recycling of the enzyme. As it was unclear whether the reaction from the previous step worked correctly (see results of table 3 above), no new substrate was added to the reaction mixture, explaining the low amounts of substrate and product in this lane.

In the next step the filter cake and the filtering cloth was directly extracted with EtOAc and the fractions are spotted on TLC plates (Figure 9).

[0146] The extraction results of the filter cake and the filtering cloth are shown in Figure 5. Lane 1 is the first extraction of the filter cake (in duplicate) with 45 ml of EtOAc. As the filter cake could not be dissolved in 45 ml of EtOAc, the remaining solids were spun down, the EtOAc phase was decanted to a fresh tube and an additional 45 ml of EtOAc were added to the sample. In this step almost all solid flakes were dissolved and the EtOAc fraction was spotted on the TLC plate (2; in duplicate). Lane C1 depicts the results obtained from the direct extraction of approx. 17.5 cm^2 of filtering cloth with 35 ml of EtOAc. As not all of the bound 10-HSA flakes could be dissolved from the tissue, the EtOAc phase was again decanted to a fresh tube and an additional 25 ml of EtOAc were added to the sample. The result of this 2nd

extraction is shown in lane C2. Lane CC is the negative control resulting from the extraction of an unused piece of cloth and is not showing any detectable spots on the TLC plate, suggesting that the cloth is stable towards EtOAc as an organic solvent.

6.) Scaled reaction from 50L fermentation of the 10-HSA production from oleic acid by the oleate hydrates from *Stenotrophomonas maltophilia*

[0147]

Table 4: Concentration of culture medium ingredients in g/l
Riesenberg MM

		Conc. g/l	handling
C-source	glucose	2	autoclave separately
salts	KH2PO4	13.3	autoclave
	(NH4)2PO4	4	
	NaOH	2.4	
	citric acid	1.7	
	MgSO4*7H2O	1.2	autoclave separately
trace elements	EDTA	0.0084	sterile filtration
	CoC2*6H2O	0.0025	
	MnCl2*4H2O	0.015	
	CuCl2*2H2O	0.0015	
	H3BO4	0.003	
	Na2MoO4*2H2O	0.0025	
	Zn(CH3COO)2*2H2O	0.013	
	Fe(III)citrat	0.1	
feed	glucose	300	

Table 5: Composition of culture medium (share in the total volume)
50 L reactor volume

- 2 L	safety volume
- 5 L	feed glucose
- 1 L	$MgSO_4$ solution
- 0.86 L	trace elements
- 0.5 L	batch glucose
- 0.05 L	Kanamycin
40.59 L	including the remaining medium ingredients

[0148] The automatic recording of the fermentation parameter are shown in Figure 10

7.) Conversion reaction

[0149] After the separation of the cells with the disc type separator 3 batches of concentrated cells (each approx. 1.5 kg, samples 1-3) were stored at -20°C.

[0150] After defrosting the concentrated cells at 4°C for 3 days samples were taken from every single batch for protein determination and the batches were combined and filled up with phosphate-citrate buffer to a final volume of 8 l. After mixing the concentrated cells homogeneously with the buffer, the cells were subsequently disrupted by high pressure homogenization (HPH). After 4 passages of homogenization (sample 4) the disrupted cells were filtered in two stages, using a (unqualified) 0.5 μm (sample 5) and a 0.2 μm (sample 6) filter cartridge.

[0151] For a further separation of the cell debris (to prevent a later extraction of cell-wall lipids), a purification of the

protein via cross-flow (x-flow) filtration was carried out. The x-flow was equipped with a 300 kDa PES filter cassette and the disrupted cells were filtered until the retentate reached a volume of 4 L, the permeate (samples 7 + 9) was collected in a 20 L canister. Subsequently dialysis was started with 13 L of phosphate-citrate buffer and ran over night. The retentate (sample 8) was kept at 4°C and the permeate was concentrated with a 30 kDa PES filter cassette to a final volume of 1 L.

[0152] Samples (Table 6) for protein and activity determination were taken from every stage of the process and analyzed by SDS-PAGE (Figure 11 und 12) or TLC. The samples for the SDS-PAGE were diluted 1:3 with 8 M urea to prevent an overload of the gel.

Table 6:

Protein samples taken during process	
sample name	description
1	concentrated cells 1st batch
2	concentrated cells 2nd batch
3	concentrated cells 3rd batch
4	after HPH
5	after 0.5 μm filter
6	after 0.2 μm filter
7	permeate start 300 kDa filtration
8	retentate after 300 kDa filtration
9	permeate end of 300 kDa filtration

[0153] The SDS-PAGE showed only small bands for the overexpression of the oleate hydratase (69 kDa) in all three batches (1-3). No difference could be detected after the HPH procedure, the filtering with 0.2 and 0.5 μm filters shows a slight decrease of unwanted proteins, respectively. Lanes 7-9 showed that all proteins, including the expressed oleate hydratase, were refrained by the filtering cassette, suggesting that an unexpected problem with the filtering cassettes existed.

[0154] As the retentate was of a dark brownish color and still containing high amounts of particulate matter a test extraction with ethyl acetate was done (Figure 13), to ensure that no remaining lipids from the bacterial cell wall will affect the following production and extraction steps.

[0155] The TLC plate didn't show any bands that would indicate lipids in the extracted samples.

To determine the enzymatic activity of the different fractions obtained during the process and to ensure an adequate conversion rate (due to low protein expression levels), 1 ml activity tests were carried with the samples 4-9 (Figure 14).

[0156] The TLC plate after the activity tests showed high conversion rates for the samples 4-6 as well as for sample 8 (in duplicate). The conversion rates for the samples 7 & 9 were (as expected from the SDS-PAGE) very low.

[0157] Based on the results from the SDS-PAGE and activity tests the conversion of the oleic acid was carried out with the retentate (sample 8) and the concentrated permeate (after 30 kDa filtration, sample not shown).

[0158] The 5 L of protein solution (4 L retentate + 1 concentrated permeate) were distributed equally into two 5 L flasks, placed on a magnetic stirring plate (400 rpm) inside an incubator and heated to the reaction temperature of 35°C. During the warming process, 2.82 L of phosphate-citrate buffer were mixed with 180 ml (≈ 160 g = 2% w/v) of oleic acid and mixed on a magnetic stirring plate. After the protein solutions reached 35°C they were filled up to 4 L volume with the mixture of buffer and substrate and incubated for 90 min under constant stirring. A sample was taken from the reaction mixture after 60 min to monitor the enzymatic activity (Figure 15). The formation of clearly visible white flakes of 10-HSA was observed during the reaction.

[0159] The results from the TLC plate (Figure 15) showed very high conversion rates from oleic acid to 10-hydroxystearic acid.

After 90 min of reaction time, the product was separated from the reaction mixture by a filtration step with miracloth fabric (4 layers). The filtering step resulted in a semi-solid filter cake and showed a good performance in the product separation.

Table 7:

Filtration results after using miracloth fabric cloth. The filter cake has been weighed and the loss of liquid using filtration has been documented.		
Preparation	Filter cake [g]	Loss of vol. [ml]
1	296.3	500
2	222.9	500

[0160] After the filtration the remaining enzyme solution was refilled to a volume of 4 L with a mixture of buffer and substrate to a final substrate concentration of 2 % (w/v) and incubated for 90 min at 35°C. During the reaction time the reaction mixture appeared as a high viscose, jellylike liquid with only very small 10-HSA flakes visible. After the reaction time the mixture was exposed to the miracloth fabric for the second filtration (Figure 16).

8.) Separation of the 10-HSA product from the recycled enzyme solution

[0161] Probes of the reaction mixture were filled in centrifuge beakers and centrifuged for 30 min at 4°C and 12200 g. After the centrifugation a semi-solid pellet appeared which showed three different layers. The supernatant remained cloudy after the centrifugation step. The resulting pellets had a weight of approx. 250 g, a 12.5 times higher weight then the inserted substrate of 20 g/L. Although the pellets still include high amounts of water and protein the centrifugation was the most successful approach for the separation of product and enzyme solution and could reduce the volume to be extracted by 75%. The pellets were subsequently dried at 50°C to further reduce the water content (see Figure 17).

[0162] 200 ml of the resulting supernatant were taken for a 2nd recycling test of the enzyme solution. 2 % (w/v) of fresh substrate were added to the solution and the sample was incubated over night at 35°C under constant stirring (250 rpm). The texture of the reaction solution did not differ from the texture of the 1st recycling step (jellylike, highly viscose liquid), suggesting that the enzyme was still active. For the separation of the product from the reaction solution a sixth approach was tested. 1.5 g (= 1% w/v) of hydrophobic Amberlite XAD2 beads were added to 150 ml of the reaction mixture and stirred for 1 h. It was thereby tested, whether the hydrophobic beads could trigger the aggregation of the 10-HSA inside the solution by acting as a nucleus for bigger aggregates. After the reaction time no bigger flakes of 10-HSA were visible and the filtering properties of the solution via miracloth fabric were as problematic as described above.

[0163] Samples were taken from the separation approach to monitor the enzymatic activity and the separation efficiency (Figure 18).

Results shown in Figure 18

[0164]

Table 8:

Sample description and respective results		
sample name	**description**	**results**
1st enzymatic reaction		
12 (0.1 g)	filter cake 1, after 1st reaction	High conversion rate, high amount of product
2nd enzymatic reaction (1st recycling step)		
13 (0.35g)	remaining mixture from cloth on funnel over night	High conversion rate, high amount of product
14 (0.35g)	corresponding filtrate of sample 13	Almost all FAs filtered from mixture
15 (0.35g)	supernatant after centrifugation	Almost all FAs filtered from mixture (slight increase towards sample 14)

(continued)

2nd enzymatic reaction (1st recycling step)		
16a (0.35g)	outer layer of the centrifugation pellet	The deeper the layer, the more product (and substrate) is seen. Correlation between product and substrate is similar and clearly on the product side
16b (0.35g)	middle layer of the centrifugation pellet	
16c (0.35g)	inner layer of the centrifugation pellet	
17 (0.35g)	upper phase of samples rested at 4°C over night	Lower amounts of product and substrate, compared to downer phase → air inside the mixture
18 (0.35g)	downer phase of samples rested at 4°C over night	High amounts of product and substrate
3rd enzymatic reaction (2nd recycling step)		
19 (0.35g)	sample from 2nd recycling over night at 35°C	2nd recycling step showed same results like the two reactions before, very god conversion rate

Description of conditions during process for the production of 10-HSA

[0165]

Table 9:

Overview of reaction conditions	
Instrument/System	**Description**
Cross Flow	Flowrate (0-100%) 0 - 1250 L h^{-1}, used range: 12 - 15%; Inlet-pressure 2.0 - 2.7 bar. Retentat- and Permeat-pressure 0 bar, Performed at 4°C
Filtration Cassette	Concentration of Pre-Filtrate from 8 L to 4 L and Diafiltration with 12 L Buffer
Filtration Cassette	Concentration of the Diafiltrate from 12 L to 1 L
Pre-Filtration	Filterholder with 1/2" TC-Clamp connection and for 5" Cartridges (0.5 m^2), Performed at Room temperature
Filter Step 1	Filtration of the 8 L Cell lysate: Pressure drop of 0.2 - 0.5 bar at the end, at a Flow rate of 250 mL min^{-1}
Filter Step 2	Filtration of the 8 L Cell lysate: Pressure Drop of 0.3 - 1.2 bar at the end, at a Flow rate of 200 mL min^{-1}
Pump and Setup	Speed (0-100%) 0 - 120 rpm. Tube with ¼" inner diameter, Filtration performed at 40 - 60 %
Cell Disruption	Flowrate of 100 L h^{-1} at a Pressure of 900 bar (single-stage Homogenizing valve); 4 recirculation (repeats of passing through homogenizer) in a 10 L stirred flask (8 L of Cell lysate) in an ice bath, Temperature kept under 40°C
Cell harvest	Harvesting with a Flow rate of 80 - 100 L h-1. Discontinuous cell ejection every 20 L. At least 5 L harvested. Final optical Density (at 600 nm) of about 250
Fermentation	Parameter of Cultivation see Chapter Fermentation 50 L
Mixing	Magnetic stirrers for 1 - 3 L and 5 - 10 L Flask; Used stirring speeds from 150 - 450 rpm

Summary

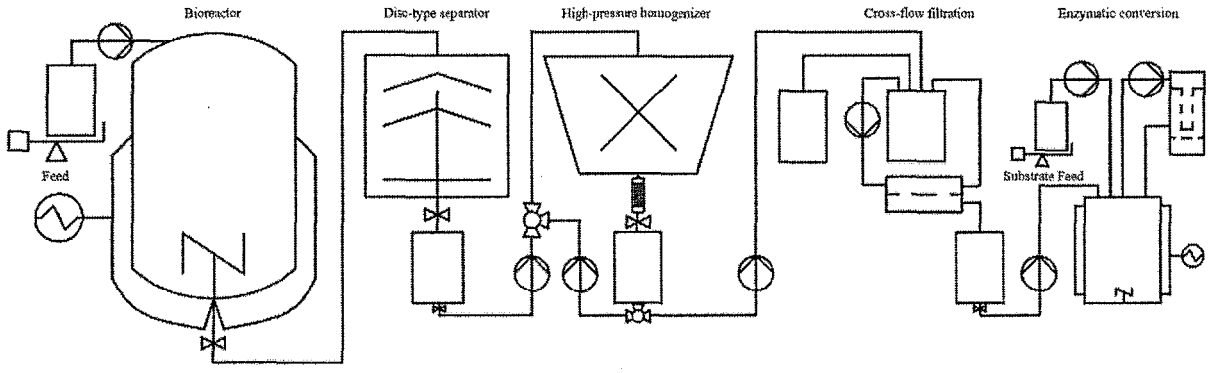
[0166] Two conversion reactions were carried out (in parallel) in a 4 L scale for 90 min at 35°C with 2% (w/w) of Substrate (OA) under constant stirring. The produced 10-HSA could be successfully filtered from the reaction mixture

and the remaining enzyme solution was used for a second (recycling) conversion reaction, without a detectable loss of enzymatic activity. It was furthermore shown that a third reaction (2nd recycling reaction) can be carried out without detectable reduction of the enzymatic activity.. In total, 320 g of oleic acid have been converted within 4 separate reactions.

Product separation:

[0167] The yield of the filtering performance using miracloth fabric was very high. TLC plate analyses show that almost all product (10-HSA) has been separated by filtration. The proportions which have passed the filter are in the lower single-digit percent area.

Scheme 5: Scheme for a technical setup in order to scale up the process of the present application The claimed process of the present application for the production of 10-HSA can be performed in a technical set-up comprising a bioreactor, a disc-type separator, a high-pressure homogenizer, a device for cross-flow filtration and enzymatic conversion.



Summary of some embodiments:

[0168]

1. Process for the cell-free enzymatic production of 10-hydroxystearic acid (10-HSA) comprising the following steps:

   1) Enzymatic hydrolysis of oil comprising at least 25% oleic acid using lipase to provide free fatty acids comprising oleic acid, and

   2) Hydration of the free fatty acids using oleate-hydratase (EC 4.2.1.53),
   wherein the oleate-hydratase is selected from *Stenotrophomonas maltophilia* or derivatives thereof having at least 10% activity when compared with wild-type enzyme under the same conditions or active fragments thereof and/or *Rhodococcus erythropolis* or derivatives thereof having at least 10% activity when compared with wild-type enzyme under the same conditions or active fragments thereof, and

   3) Separation of 10-HSA in form of a filter cake from the reaction mixture, and

   4) Purification of 10-HSA from the separated filter cake,

   and wherein, optionally, the lipase and/or the hydratase used in step 1) and /or step 2) is/ are recycled and/or immobilised.

2. The process according to embodiment 1, wherein said oil is selected from the group comprising renewable/re-growing feedstocks.

3. The process according to embodiment 1 or 2, wherein said feedstocks are selected from the group comprising animals, plants and microorganisms.

4. The process according to any of the preceding embodiments, wherein said oil comprises triglycerids with an oleic acid content ≥40 %, preferably ≥50 %, more preferably ≥70%.

5. The process according to any of the preceding embodiments, wherein said oil is a plant oil selected from the group comprising vegetable oil, castor oil, tree borne oil, olive oil, mustard oil, linseed oil, canola oil, coconut oil, coriander oil, corn oil, cottonseed oil, hazelnut oil, neem oil, palm oil, peanut oil, rapeseed oil, rice bran oil, safflower oil, soybean oil, sunflower seed oil, and mixtures thereof.

6. The process according to any of the preceding embodiments, wherein said oil is an animal oil selected from the group comprising tall, fish and crustacean oil.

7. The process according to any of the preceding embodiments, wherein said oil is a microbial oil.

8. The process according to embodiment 7, wherein said microbial oil is derived from bacteria, yeast, algae and/or fungi.

9. The process according to any of the preceding embodiments, wherein the enzymatic hydrolysis of oil using lipase (step 1) and the hydration using oleate hydratase (step 2) can be carried out sequentially or simultaneously.

10. The process according to any of the preceding embodiments, wherein said lipase is selected from the group comprising mono-, di- or triglyceride lipase.

11. The process according to any of the preceding embodiments, wherein said lipase is a lipase selected from the EC class of hydrolases (EC class 3.), preferably from the EC class of esterase enyzmes acting on ester bonds (EC class 3.1), more preferably from the class of carboxylic-ester hydrolases (EC class 3.1.1).

12. The process according to any of the preceding embodiments, wherein said lipase is a lipase selected from the EC class EC 3.1.1.3.

13. The process according to any of the preceding embodiments, wherein said triglyceride lipase is lipase selected from the group comprising *Candida rugosa* lipase, lipase from porcine pancreas, lipase from *Rhizopus oryzae or* lipase from Pseudomonas sp..

14. The process according to any of the preceding embodiments, wherein said enzymatic hydrolysis is carried out in an aqueous system.

15. The process according to any of the preceding embodiments, wherein said aqueous system comprises at least one buffer and/or at least one solvent, mixtures of solvents and/or mixtures of buffer(s) and solvent(s).

16. The process according to any of the preceding embodiments, wherein said buffer is selected from Tris-HCl buffer, phosphate-citrate-buffer, 3-(N-morpholino)propanesulfonic acid (MOPS) buffer.

17. The process according to any of the preceding embodiments, wherein the concentration /amount of said buffer is in the range of 10 mM to 100 mM.

18. The process according to any of the preceding embodiments, wherein said enzymatic hydrolysis is carried out at a temperature ranging from 10°C to 60 °C.

19. The process according to any of the preceding embodiments, wherein said enzymatic hydrolysis is carried out for a period of 15 to 300 minutes, preferably 30 to 180 minutes, more preferably 60 - 90 min

20. The process according to any of the preceding embodiments, wherein after the enzymatic hydrolysis (step 1) said free fatty acids are optionally separated from the reaction mixture by at least one washing/purification step.

21. The process according to any of the preceding embodiments, wherein after the enzymatic hydrolysis (step 1),

said free fatty acids are separated from glycerol in at least one washing/purification step by extraction or phase separation.

22. The process according to any of the preceding embodiments, wherein after the enzymatic hydrolysis (step 1) said free fatty acids are optionally separated from the reaction mixture by at least one washing/purification step using at least one organic solvent and/or an aqueous system.

23. The process according to embodiment 22, wherein said organic solvent is selected from the group ethylacetate, hexane, toluene, methyl isobutyl ketone (MIBK), methanol, ether.

24. The process according to any of the preceding embodiments, wherein said hydration using oleate-hydratase (step 2) is carried out in an aqueous system.

25. The process according to any of the preceding embodiments, wherein said aqueous system for the hydration comprises buffer, free fatty acids as substrate, oleate hydratase and/or emulsifier.

26. The process according to any of the preceding embodiments, wherein said buffer is selected from Tris-HCl buffer, phosphate-citrate-buffer, 3-(N-morpholino)propanesulfonic acid (MOPS) buffer, 2-(*N*-morpholino)ethanesulfonic acid (MES) buffer, piperazine-N,N'-bis(2-ethanesulfonic acid) (PIPES); 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES); Sørensen's phosphate buffer (Stock solutions: A 0.2 M NaH_2PO_4, B 0.2 M Na_2HPO_4).

27. The process according to any of the preceding embodiments, wherein said hydration is carried out at a temperature ranging from 10°C to 50°C, preferably 20 -40 °C, more preferably 25 - 35 %.

28. The process according to any of the preceding embodiments, wherein said hydration is carried out for a period of 10 to 300 minutes, preferably 30 to 180 minutes, more preferably 60 to 90 minutes.

29. The process according to any of the preceding embodiments, wherein the hydration is carried out under constant mixing, wherein the speed is in the range of 250 to 750 rpm.

30. The process according to any of the preceding embodiments, wherein after the hydration (step 2) 10-HSA is separated from the reaction mixture in form of a filter cake using filtration and/or centrifugation and/or using chromatographic methods.

31. The process according to any of the preceding embodiments, wherein after the hydration (step 2), 10-HSA is prepared from the filter cake using extraction with at least one organic solvent and/or using phase generation.

32. The process according to any of the preceding embodiments, wherein after the hydration (step 2), 10-HSA is prepared from the filter cake using extraction with at least one organic solvent, wherein said organic solvent is selected from the group comprising ethyl acetate, hexane, methanol, ether, methyl-isobutyl-ketone, toluene.

33. The process according to embodiments 31-32, wherein the concentration of said organic solvent is between 80% w/v to 100% w/v.

34. The process according to embodiments 31-33, wherein the concentration of said organic solvent is $\leq$ 90% w/v.

35. The process according to any of the preceding embodiments, wherein after the extraction of 10-HSA from the filter cake, purification of 10-HSA is performed (step 4) using phase separation.

36. The process according to any of the preceding embodiments, wherein said purification of 10-HSA from the filter cake (step 4) is performed by phase separation, wherein the dissolved 10-HSA is separated from the residual fraction/reaction mixture comprising water and enzymes in form of a filter cake using filtration and/or centrifugation and/or using chromatographic methods, and wherein, optionally, 10 HSA flocks are separated from the mixture by filtration or gravimetric solid-liquid-separation.

37. The process according to any of the preceding embodiments, wherein said purification of 10-HSA from the filter cake (step 4) is performed by phase separation, wherein 10-HSA is dissolved in at least one organic solvent.

38. The process according to embodiment 37, wherein said organic solvent is selected from the group comprising ethyl acetate, hexane, methanol, ether, methyl-isobutyl-ketone, toluene.

39. The process according to embodiments 37-38, wherein the concentration of said organic solvent is between 80% w/v to 100% w/v.

40. The process according to embodiments 37-39, wherein the concentration of said organic solvent is ≤ 90% w/v.

41. The process according to embodiments 37 to 40, wherein the organic solvent is removed by evaporation, distilling, gassing with nitrogen and/or phase separation.

42. Composition or product comprising 10-HSA obtainable by the process according to any of the preceding embodiments, wherein, preferably, said composition or product is selected from specialty oleochemicals, chemical performance additives, cosmetics, cosmetic additives, in particular lubricants.

Claims

1. Process for the cell-free enzymatic production of 10-hydroxystearic acid (10-HSA) comprising the following steps:

1) Enzymatic hydrolysis of oil comprising at least 25% oleic acid using lipase to provide free fatty acids comprising oleic acid, and
2) Hydration of the free fatty acids using oleate-hydratase (EC 4.2.1.53),
wherein the oleate-hydratase is selected from *Stenotrophomonas maltophilia* or derivatives thereof having at least 10% activity when compared with wild-type enzyme under the same conditions or active fragments thereof and/or *Rhodococcus erythropolis* or derivatives thereof having at least 10% activity when compared with wild-type enzyme under the same conditions or active fragments thereof, and
3) Separation of 10-HSA in form of a filter cake from the reaction mixture, and
4) Purification of 10-HSA from the separated filter cake,

and wherein, optionally, the lipase and/or the hydratase used in step 1) and /or step 2) is/ are recycled and/or immobilised.

2. The process according to claim 1, wherein said oil is selected from the group comprising renewable/regrowing feedstocks.

3. The process according to claim 1 or 2, wherein said feedstocks are selected from the group comprising animals, plants and microorganisms.

4. The process according to any of the preceding claims, wherein said oil comprises triglycerids with an oleic acid content ≥40 %, preferably ≥50 %, more preferably ≥70%.

5. The process according to any of the preceding claims, wherein said oil is a plant oil selected from the group comprising vegetable oil, castor oil, tree borne oil, olive oil, mustard oil, linseed oil, canola oil, coconut oil, coriander oil, corn oil, cottonseed oil, hazelnut oil, olive oil, neem oil, palm oil, peanut oil, rapeseed oil, rice bran oil, safflower oil, soybean oil, sunflower seed oil, and mixtures thereof.

6. The process according to any of the preceding claims, wherein said oil is an animal oil selected from the group comprising tall, fish and crustacean oil.

7. The process according to any of the preceding claims, wherein said oil is a microbial oil.

8. The process according to claim 7, wherein said microbial oil is derived from bacteria, yeast, algae and/or fungi.

9. The process according to any of the preceding claims, wherein the enzymatic hydrolysis of oil using lipase (step 1) and the hydration using oleate hydratase (step 2) can be carried out sequentially or simultaneously.

10. The process according to any of the preceding claims, wherein said lipase is selected from the group comprising

mono-, di- or triglyceride lipase.

11. The process according to any of the preceding claims, wherein said lipase is a lipase selected from the EC class of hydrolases (EC class 3.), preferably from the EC class of esterase enzymes acting on ester bonds (EC class 3.1), more preferably from the class of carboxylic-ester hydrolases (EC class 3.1.1).

12. The process according to any of the preceding claims, wherein said triglyceride lipase is lipase selected from the group comprising *Candida rugosa* lipase, or lipase from porcine pancreas, lipase from *Rhizopus oryzae or* lipase from Pseudomonas sp..

13. The process according to any of the preceding claims, wherein said enzymatic hydrolysis is carried out in an aqueous system.

14. The process according to any of the preceding claims, wherein after the enzymatic hydrolysis (step 1) said free fatty acids are optionally separated from the reaction mixture by at least one washing/purification step.

15. The process according to any of the preceding claims, wherein after the hydration (step 2) 10-HSA is separated from the reaction mixture in form of a filter cake using filtration and/or centrifugation and/or using chromatographic methods, and wherein, optionally, 10 HSA flocks are separated from the mixture by filtration or gravimetric solid-liquid-separation.

16. The process according to any of the preceding claims, wherein after the extraction of 10-HSA from the filter cake, purification of 10-HSA is performed (step 4) using phase separation.

17. A composition or product comprising 10-HSA obtainable by the process according to any of the preceding claims, wherein, preferably, said composition or product is selected from specialty oleochemicals, chemical performance additives, cosmetics, cosmetic additives, in particular lubricants.

Figure 1:

```
Rer   ---------------------------------------------------------MSS 3
Sma   ---------------------------------------MYYS--SGNYEAFARPRKPAG 19
ohyA  MNPITSKFDKVLNASSEYGHVNHEPDSSKEQQRNTPQKSMPFSDQIGNYQR-NKGIPVQS 59
Mca   ---------------------------------------MYYS--NGNYEAFARPKKPEG 19
Bbr   ---------------------------------------MYYS--SGNYEAFARPKKPAG 19
Ckr   MSIYQSTY-----PTDQFG-------------------NRFVQNDMGKYIE-TRPEVSSD 35
Oan   ---------------------------------------MYRS--NGNFEAYARPPKPEG 19
Mod   MKVDTQKFDKILETSSKYGHVNQAPNGNTEPPINTAQRSMPFADEPGNYQR-NKGIPSKD 59
Sau   ---------------------------------------MYYS--YENYEAFARPKKPEN 19
Cgl   MSTINSKFDKVLNASDQFGNVNHEPDSSKEVQINTPEKTMPFSDQIGNYQR-NKGIPLQS 59
Cal   MGKITEKFDKVLNASPFPGHIDHAPDASKEVVRNSKDQPMPFADLKGNYQR-NKGIPSKS 59
Lac   ---------------------------------------MYYS--NGNYEAFADPKKPAG 19

Rer   NLSHKAYMIGAGIGNLSAAVYLIRDGEWNGEDITIMGLDMH--GANDGESAATFQHQYGH 61
Sma   VDGKRAWFVGSGLASLAGAAFLIRDGRMAGERITILEQQHIPGGALDG------------ 67
ohyA  YDNSKIYIIGSGIAGMSAAYYFIRDGHVPAKNITFLEQLHIDGGSLDG------------ 107
Mca   VDNKSAYLVGSGLASLAAASFLIRDGQMKGENIHILEELDLPGGSLDG------------ 67
Bbr   VDSKHAYIIGTGLAALSSACYLVRDGQMPGDHIHILEKDPVPGGACDG------------ 67
Ckr   IKDRKAYFVGSGIGNLIAAGYLLRDGQMPGENITFLEQLDVAGGSFDG------------ 83
Oan   VDGKTAYFVGAGLASLAGAAFLIRDAQLSGENIIIFEELALPGGSMDG------------ 67
Mod   FSQSKVYIIGSGIAGMAAAYYFIRDGRIPAANITFLEQLSIDGGSLDG------------ 107
Sau   VENKSAYLIGSGLASLAAACFLIRDGQMEGSKIHILEELPKAGGSLDG------------ 67
Cgl   YENSKIYIVGSGIAGMSAAYYFIRDGRVPGKNIIFLDQLNVEGGSLDG------------ 107
Cal   FKDSKVYIVGTGIAGLSAAYYFIRDGHIPGENIIFLDKIAIEGGSLDG------------ 107
Lac   VDKKSAYIIGSGLAGLSTAVFLVRDAQMKGENIHILEELPVAGGSLDG------------ 67
                ::::*:*:. :  * :::**..  .  * ::       *: **

Rer   RELGNDAGFINRGGRMLNEETYENLWDVLSAVPSLDNPG-KSVTDDILDFDHAHPTHDVA 120
Sma   -LKVPEKGFVIRGGREM-EDHFECLWDLFRSIPSLEIED-ASVLDEFYWLNKDDPNYSLQ 124
ohyA  -AGNPTDGYIIRGGREM-DMTYENLWDMFQDIPALEMPAPYSVLDEYRLINDNDSNYSKA 165
Mca   -ILNPERGYIMRGGREM-ENHFECLWDLFRSVPSLEVED-ASVLDEFYWLNKEDPNYSKC 124
Bbr   -LDIPGLGYVMRGGREM-DNHFEVMWDLFRSIPSIETEG-VSVLDEYYWLNKEDPNYSLC 124
Ckr   -AGNNQEGFIARGGREM-GQHFECFWDIMKDVPAIEMPAPHTVLDEFNKVNEEDPNISNC 141
Oan   -ILDEHKGFIVRGGREM-EAHFETLWDLFRSIPSLDTPD-ASVLDEMYWLHKKDPSRNPC 124
Mod   -AGNAQEGYIVRGGREM-DMTYENLWDIFQDIPALELPKPYSVLDEYRLINDNDPNYSKS 165
Sau   -ENMPLKGYVVRGGREM-ENHFECLWDLFRSIPSLEIDH-ASVLDEFYWLNKEDPNYSRC 124
Cgl   -AGNAKDGYIIRGGREM-DMTYENLWDMFQDIPALELPAPYSVLDEYRLVNDNDPNYSKA 165
Cal   -SGNAKDGYLIRGGREL-EMNYENLWDIFQDIPALEMPAPYSVLDEFRLLNDNDPNYSKA 165
Lac   -ADRPNAGFVVRGGREM-ENHFECLWDMYRSIPSLEVPG-ASYLDEYYWLDKEDPNSSNC 124
          *:: **** :     :* :**:   :*:::      :  *:    ... .  .  .

Rer   RLIDRDGIRNKGENDYKHMQFDNKDRYLLTKLMTMPESDEAKLDDISIEQWFEETPHFFT 180
Sma   RATINRGEDAHTDGL---FTLTEQAQKDIIALFL---ATRQEMENKRINEVLGR--DFLD 176
ohyA  RLINNKGEIK-DFSK---FGLNKMDQLAIIRLLL---KNKEELDDLTIEDYFSE--SFLK 216
Mca   RVIENRGQRLESDGK---MTLTKKANKEIIQLCL---MKEEQLNDVKISDVFSK--DFLD 176
Bbr   RATKDLGKDAGLKGK---FGLSDKASMEIMKLFF---TPDEDLYDKPITDFFDD--EVLN 176
Ckr   RIISNQAQTRLKNPK---LELSKKAQLQIVKLLL---AKEEDTYYKTIEDWFGK--DFLE 193
Oan   RATEGRGDPIPHMAD---LTLTPKAVEEMLKLAL---TPESALDDKRIDECFGE--EFFA 176
Mod   RFIHQLGEIK-DFSQ---FGLSKKDQMALIKLLL---KRKEELDDITIEQYFHS--SFLE 216
Sau   RVIEKQGQRLVTDGD---FTLTKTAIKEILDLCL---TNEEDLDDVKITDVFSD--DFFN 176
Cgl   RLIHNQGQIK-DFSK---FGLEKKDQLAIVKLLL---KKKEELDDLTIEDYFSE--SFLN 216
Cal   RLIHNNGEIQ-DFSK---FGLDKLDQLAIVKLLL---KKKEELDDVTVESYFSD--SFFK 216
Lac   RLIYNRGDRLPSDGQ---YGLGKCA-NEIVKLIM---TPEKEIEGQTIEEFFSD--EFFK 175
      *    .              :       :  *                 : . :     .:

Rer   ---GNALNTFVDSNVLLSIVVHHQPHYHAQKEN-EGVFWGYCLFPRKDGDYVKKPFIEMT 432
Sma   KVVTGGIVSVRDSRWLMSWTVNRQPHFKNQPKD-QIVVWVYSLFVDTPGDYVKKPMQDCT 430
ohyA  KTVTGGIITITDSNWLMSFTCNRQPHFPEQPDD-VLVLWVYALFMDKEGNYIKKTMLECT 475
Mca   RTVTGGIVTVDDSNWQLSFTLNRQQQFKNQPDD-QVSVWIYALYSDERGERTNKTIVECS 431
Bbr   KVVTGGIVTVQDSNWLMSWTLNRQQQFRDQPKD-QLCVWVYGLFPDKPGNYVKKPMTECT 466
Ckr   RAVTAGIITAQDSPWLCSLTVHRQPQFPGQQDG-LCVAWAYGLHWWKKGTVTGKPMLECT 446
Oan   RIVTGGPCNFKDSNWLYGYTMSRQPHFKAQDESQKLVVWLYGLFSDKPGNYVKKKIRECA 432
Mod   KTVTGGIITITDSNWLMSFTCNRQPHFPTQPDD-ILVLWVYALFMDKDGNYVKKTMPACT 475
Sau   KTVTGGIITINDSAWQISFTINRQQQFKDQPKN-EISTWIYALYSDVNGDYIKKPITECS 431
Cgl   RTATGGIITITDSNWVMSFTCNRQPHFPTQPDD-ILVVWVYALLMDKEGNYIKKTMPQCT 475
Cal   KSATGGIVTITDSNWLMSFTINRQPHFPEQPDD-ILVIWVYALFMDKNGNYSKKTMPQCT 475
Lac   KVNTGGIITIVDSNWELSFTIHRQPHFKSQNPD-QIVVWIYALYSDTEGNYIKKRIVDCT 430
          .  .  **    . .  :* ::  *         * * *     *     * :   :

Rer   GREMLEETLGHLEALDESGTLAARRQEIMDSVVNSIPSHMPYASALFNRRAVGDRPLVVP 492
Sma   GEEITREWLYHLGVPVEEIDELAA-----T-GAKTVPVMMPYITAFFMPRQAGDRPDVVP 484
ohyA  GDEILAELCYHLGIEDQL-ENVQK-------NTIVRTAFMPYITSMFMPRAKGDRPRVVP 527
Mca   GKEICEEWLYHMGVPEEKISALAA-------ECNTIPSYMPYITAYFMPRKEGDRPLVVP 484
Bbr   GEEICEEWLYHMGVPTDKIESLAK-----H-HANTVPVMMPYITAFFMPRAAGDRPDVVP 520
Ckr   GEEILREFCYHFGVVDVE--KTIK-------HTKVRLAVMPYITSEFVPRGAGDRPDPVP 497
Oan   GAELCEEWLFHMGVPVEDIPALAR-----R-SASTVPCNMPYITSYFMPRAMGDRPLVVP 486
Mod   GNEILAELCYHLGIIDSL-NQVVE-------NTIVRTAYMPYITSMFMPRAQGDRPQVVP 527
Sau   GNEICQEWLYHLGVPTDKIEDLAK-----H-ASNTIPVYMPYITSYFMTRAIGDRPLVVP 485
Cgl   GNEILAELCYHLGITDQL-DNVTE-------NTIVRTAFMPYITSMFMPRAMGDRPRVVP 527
Cal   GNEVLAELCFHIGLEDQI-ATIIK-------NTIVKTSFMPYITSMFMPRAAGDRPEVVP 527
Lac   GKEIAEELLYHLGVPESQISELAS-----EENMNTVPVYMPYITSYFMPRRDGDRPDVVP 485
      * *:  *    *:                          *** :: *  *  ****  **

Rer   KHSKNLAFISQFAEL-PFDMVFTEQYSVRCAQVAVYKFLGIPEDKLTKMHHYEKDPKVLA 551
Sma   DGAVNFAFIGQFAESKQRDCIFTTEYSVRTPMEAVYTLLDIERGVPE-VFNSTYDVRSLL 543
ohyA  EGCKNLGLVGQFVET-NNDVVFTMESSVRTARIAVYKLLNLNKQVPD-INPLQYDIRHLL 585
Mca   HGSKNIAFIGNFAET-ERDTVFTTEYSVRTAMEAVYKLLEVDRGVPE-VFASVYDVRILL 542
Bbr   DGAVNFAFLGQFAET-PRDTIFTTEYSMRTGMEAVYTLLGVDRGVPE-VWGSVYDVRNLL 578
Ckr   AGSTNLGFTGQFVET-PDDCVFTTEGSARTGQMAVYGLLNLKRDIPP-IYPVQYDIRALL 555
Oan   DGSKNLAFIGNFAET-EKDTVFTTEYSVRTAMEAVYTLFNVDRGVPE-VFASSFDVRVLM 544
Mod   EGCLNLGLIGQFVET-HNDVVFTMESSVRTGRQAVYQLLNLNKQVPD-IFPLQYDIRHLL 585
Sau   HQSQNLAFIGNFAET-ERDTVFTTEYSVRTAMEAVYQLLNIDRGIPE-VINSTFDLRVLM 543
Cgl   EGCTNLGLVGQFVET-NNDVVFTMESSVRTARIAVYNLLNLNKQVPD-INPLQYDIRHLL 585
Cal   NGSKNLGLVGQFVET-HNDVVFTVDASIRTARIAVYKLLNLNKQVPD-IAAGQYDIRQLL 585
Lac   EGSINLAFIGNFAESPTRDTVFTTEYSVRTAMEAVYTLLNVDRGVPE-VFDSIYDIRQLL 544
        . *:.: .:*.*    * :** : * *     *** :: : .       :     * : *

Rer   TNFWYMWETTFAFKRVSSAMELRRYMNRMILEFSRIQTLAGVTRSPYNQYESIILPMRTF 240
Sma   SNFWLYWRTMFAFEEWHSALEMKLYLHRFIHHIGGLPDFSALKFTKYNQYESLVLPLVKW 236
ohyA  SNFWTFWRTMFAFENWHSLLELKLYMHRFLHAIDGLNDLSSLVFPKYNQYDTFVTPLRKF 276
Mca   SNFWIYWKTMFAFEPWHSAMEMRRYLMRFIHHIGGLADFSALKFTKFNQFESLVMPLIEH 236
Bbr   SNFWLYWRTMFAFENWHSALEMKLYIKRYIHHIGGLPDFSALRFTRYNQYESMILPMVKY 236
Ckr   SNFYTLWRSMFAFQDYQSLTEMKRYFHRFLQYLPGFSDFSCLRFSKYNQFTSFIEPLRNF 253
Oan   SNFWLYWATMFAFEPWASAMEMRRYILRFVHHIATLADLSSLRFTRYNQYESLIMPLVAW 236
Mod   SNFWTFWRTMFAFENWHSLLEFKLYMHRFLHDIDGLKDLSALVFPRYNQYDTFVAPLRNH 276
Sau   SNFWIYWKTMFAFEPWHSAMEMRRYLMRFVHHIGGLADFSALKFTKYNQYESLVLPMVEY 236
Cgl   SNFWFFWRSMFAFENWHSLLELKLYMHRFLHAIDGMKDFSCLVFPKYNQYDTYVTPLKNF 276
Cal   SNFWTLFRTMFAFENWHSLLECKLYMHRFLHRIDGFNDLSCLVFPKYNQHDTFVKPLTDH 276
Lac   TNFWTYWSTMFAFEKWHSLAEMRRYAMRFIHHIDGLPDFTALKFNKYNQYESMVKPLLAY 235
      :**:  : : ***:    *  * : *  * :  :  :  :: :    :**. : : *:

Rer   LEGKGVKFVNELKITEFVFKDTP----LR----------------------------DEI 268
Sma   LQDQGVVFQYGTEVTDVDFDLQP----------------------------------DRK 262
ohyA  LQEKGVNIHLNTLVKDLDIHINT----------------------------------EGK 302
Mca   LKAKNVTFEYGVTVKNIQVECSK----------------------------------ESK 262
Bbr   LESHGVEFRYNTKVENVEFAIGGGDGPKREHTGVGQDTIQKIQATSGFFKRNPASTPTKK 296
Ckr   LKEKGVKFQYGTCVKDLDIDVKG----------------------------------SSF 279
Oan   LEGKGVRFQYDTQVENIEVETAG----------------------------------GNK 262
Mod   LTELGVQIRLDTLVHDVDLHSTT----------------------------------AGK 302
Sau   LKSHGVQFEYDVKVEDIKVDVTT----------------------------------SQK 262
Cgl   LVEKGVQIQFNTLVKDLDIHINT----------------------------------EGK 302
Cal   LKSKGVKIQFNTFVKDLEVQINT----------------------------------EGK 302
Lac   LKDHGVQFEYDCHVKNVEVDHEG----------------------------------DSK 261
      *    * :      : :. .

Rer   IVTGLDYENVRTGEKGRIDVAEGDFVFDTNGSITDSSSIGDLDTPIVED-----MRYAPS 323
Sma   QATRI--HWMHDGVAGGVDLGADDLLFMTIGSLTENSDNGDHHTAARLN-----EGPAPA 315
ohyA  VVEGI--ITEQDGKEVKIPVGKNDYVIVTTGSMTEDTFYGNNKTAPIIGIDNSTSGQSAG 360
Mca   VAKAI--DIVRRGNEESIPLTENDLVFVTNGSITESTTYGDNDTPAPPT-----SKPGGA 315
Bbr   LAVRI--DVSQEGEKSSIDLTENDLVFITNGGCVENSTMGSQNSPAAWNP---DLKPGGG 351
Ckr   TVTGI--VTNKE----TIPTRSQDIVIVTNGSLTESTGYGDMNTVPEFK-----KTPGPA 328
Oan   LARRL--VMTVGGEPKTIELTENDVVFVTNGSITESSTFGDNDHPAPIE-----TGHGGA 315
Mod   LVKGL--LVNQGGQETRIEMNEQDFVVITTGSMTEDTSYGTNTTVPIPKVDNTTSGKSPG 360
Sau   IAREI--LIHRHGKAESIKLTVDDLVFVTNGSITESSTYGDNDTPAPPT-----DELGGS 315
Cgl   TVEGI--ITEQNGEEVKIPISKEDYVIVTTGSMTESTFYGDNNTVPEVTIDNSSAGQSAG 360
Cal   VVKGI--ITQQEDKEVTIAVTENDFVIVTTGSMTEDTHYGDNINAPIVAIDDIKSGESDG 360
Lac   IAKKI--VMTQNGKDKEIDLTHNDIVFVTNGSITESSTYGDQNTPAPIT-----NAKGDS 314
        .  :              :       * :. * *. .:.:  *              . .

Rer   ALLWKQATEHFYDLGNPDKFFGDRAQSEWT-SFTVTTSSHELINEIS---RITKQLP--- 376
Sma   WDLWRRIAAKDDAFGRPDVFGAHIPETKWE-SATVTTLDARIPAYIQ---KIAKRDPFSG 371
ohyA  WKLWKNLAAKSEIFGKPEKFCSNIEKSAWE-SATLTC---KPSALIDKLKEYSVNDPYSG 416
Mca   WQLWENLSTQCEEFGNPAKFYKDLPEKSWFVSATATTNNKEVIDYIQ---KICKRDPLSG 372
Bbr   WDMWRRIAEQDPSFGHPEKFCSDPNATKWM-SATVTTLDDEIPPYIQ---KICKRDPFSG 407
Ckr   WSLWKNIAEKAPNCGRPERFCSDPESTVWE-SISFNFYDGYDNPFTQKLKELTHRDVFNG 387
Oan   WTLWKNLAAQHPAFGRPEKFCEDIPDANWTISATVTLLDDKIVPWIE---KMTGRDPRDG 372
Mod   WSLWKNLAAKSPVFGRPEKFCSNIEKSSWE-SATLTC---KPSPLIDKLKEYAVNDPYSG 416
Sau   WTLWKNLARQSPEFGNPDKFCQNIPQKSWFVSATSTTNNKDIIDTIE---SICKRDPLAG 372
Cgl   WKLWKNLAAKSEVFGKPEKFCSHIEKSSWE-SATLTC---RPSAFTEKLKELCVNDPYSG 416
Cal   WQLWKNLATKSIEFGKPEKFYSSVKKSSWE-SATLTC---RPSAFTEKIKEYCVNDPYSG 416
Lac   WKLWENLAKQDPAFGHPDVFCENLPERSWFVSATATLENKKLAPYFE---RLTKRSLYDG 371
        :*.. : :     *.*  *          *  * : .             .       .

Rer   KAAVTMFR---------------------------------------------------- 559
Sma   AATGRLRDGKELGIPGPV-FLRN----LLMNKLDKTQIGGLLREFKLVQED--------- 589
ohyA  KAAKTLNDDKPFVGEG-----------LLRKVLKGTYFEHVLPAGAAEEEEHESFIAEHV 634
Mca   HALSVLNDGKKLDEIDMP-FYERLVEKRLLKKASGTFIEELLEEANLI------------ 589
Bbr   NATVKLRDGAPVTDMKLN-FIEKAVVKKVLKKLDGTDIATLLREYHVI------------ 625
Ckr   HSASAMNDG-KLPGEK-----------LLRKFLKNTYYENIIPKGSSH------------ 591
Oan   SALYYLNDRKKLDEIQLP-FVARLLGKVAMKKIEGTYLEELLKDAKLV------------ 591
Mod   KAAHALNDDQPIVGEA-----------LLRKFLQGTYYEHILPTVEKRKDDQESFFVEQY 634
Sau   DAIYELNDHQDLREITKDSKIQKLALAGFLKKIKGTYIESLLKEHKLL------------ 591
Cgl   KATQALNDYKPFLGEG-----------ILRKILKGTYFEHILVNRPEEKEEHESFL---- 630
Cal   KAAKALNDYKPFPGES-----------VLKRVLKNTYFEHILPEGVEDEEQHDSFLTEQL 634
Lac   RAMYYMSDKKKLADQDMP-LPEKLAVKTGMRKIKKTWVEELLKEANLV------------ 591
       :   :

Rer   ------------
Sma   ------------
ohyA  NKFREWVKGIRG 646
Mca   ------------
Bbr   ------------
Ckr   ------------
Oan   ------------
Mod   EKAKDWFKKLIG 646
Sau   ------------
Cgl   TRFQEWVKGVKD 642
Cal   EKLKGWAKELTH 646
Lac   ------------
```

Figure 2:

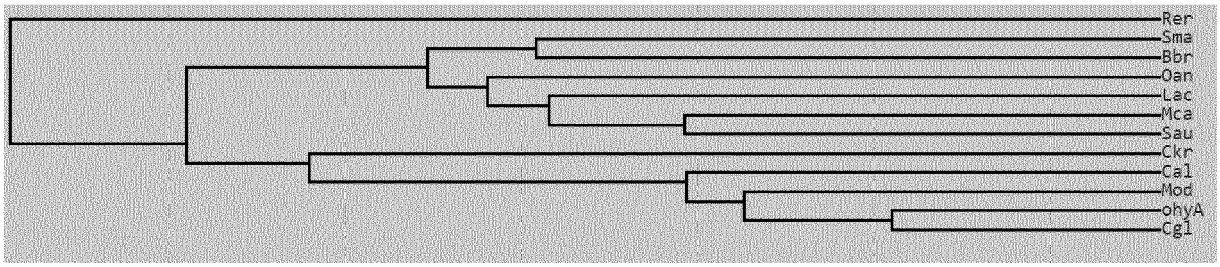


Figure 3:

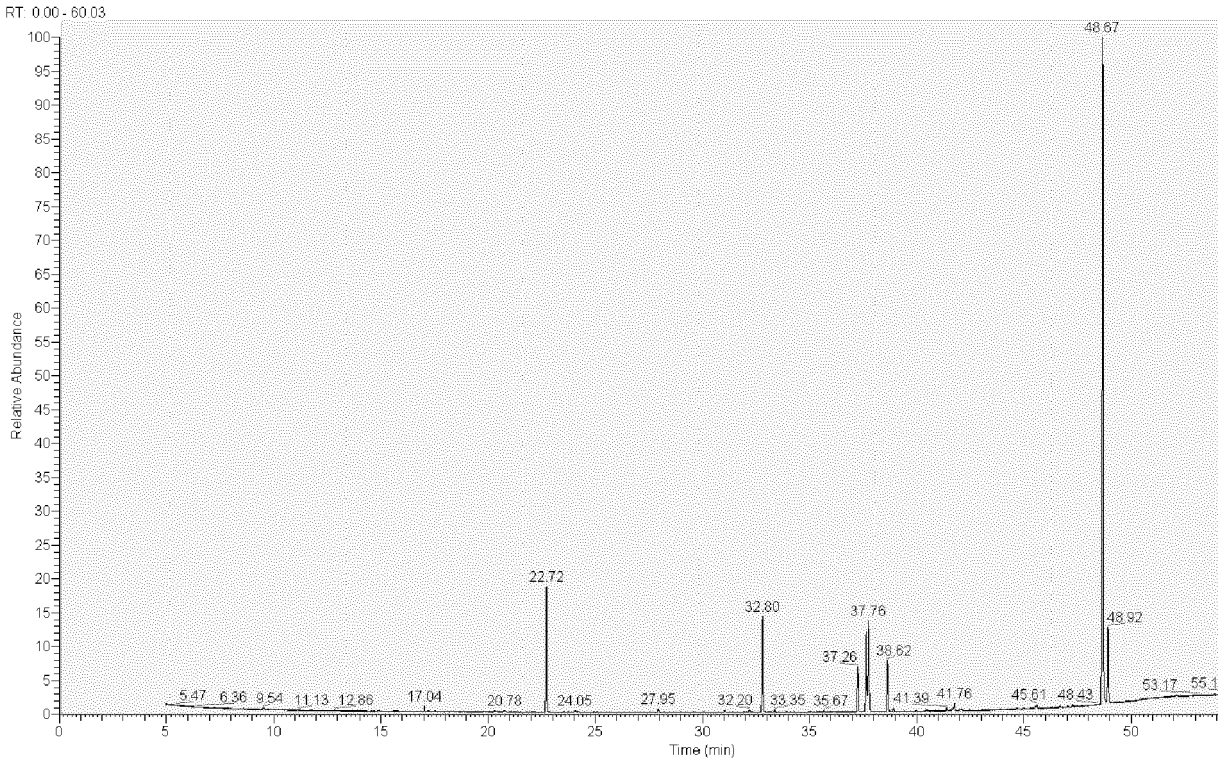


Figure 4:

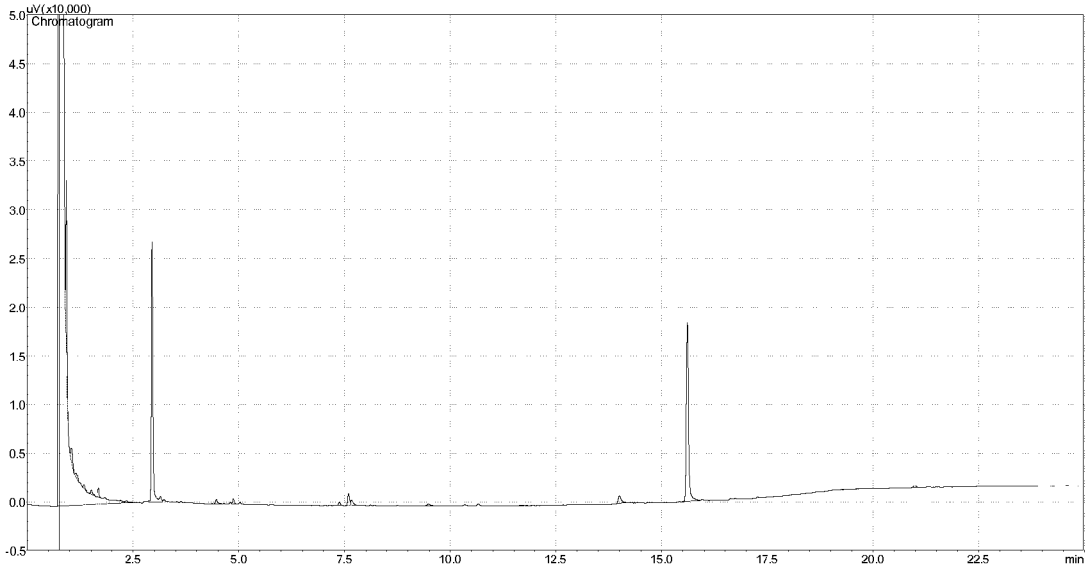


Figure 5:

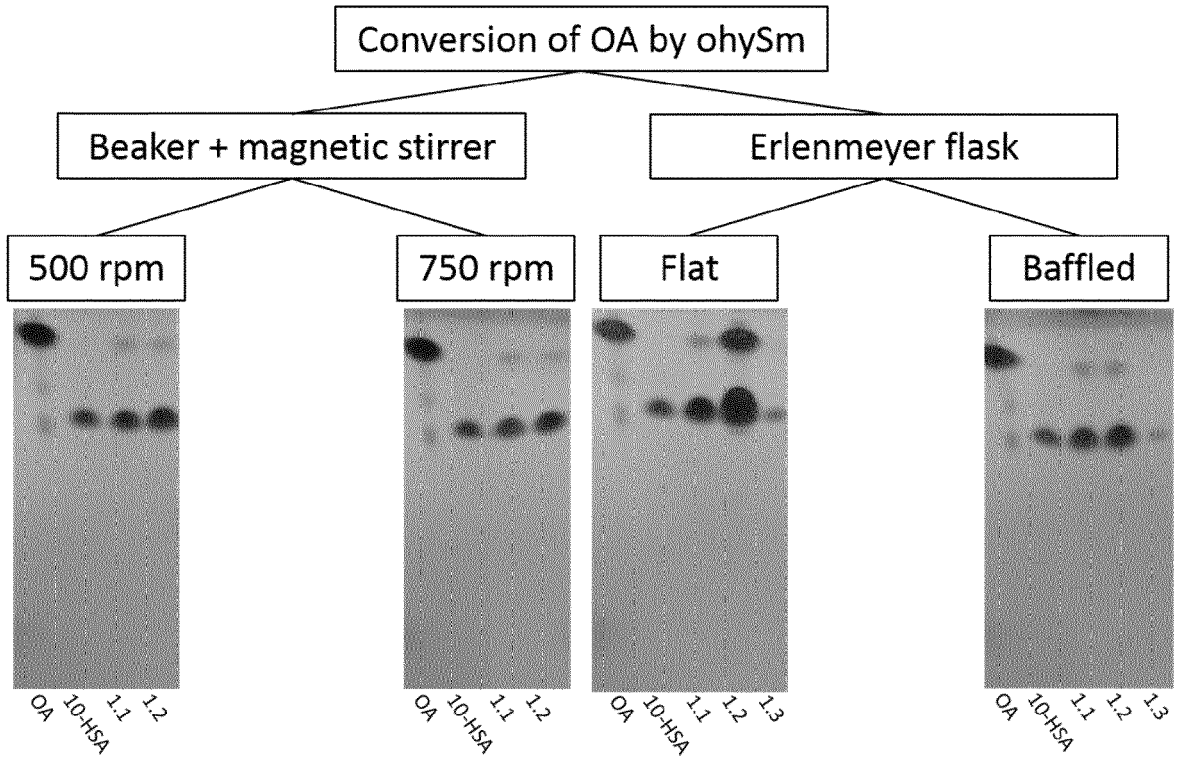


Figure 6:

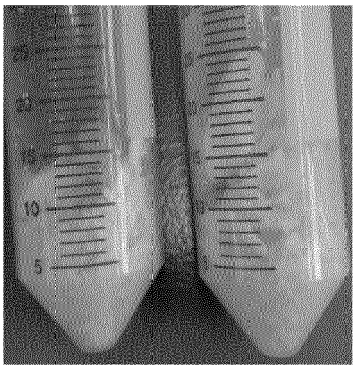


Figure 7:

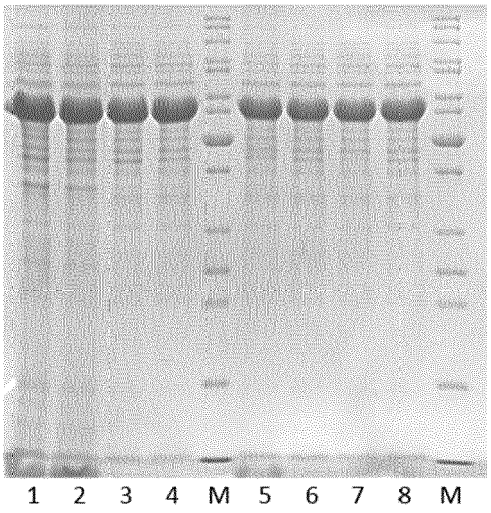


Figure 8:

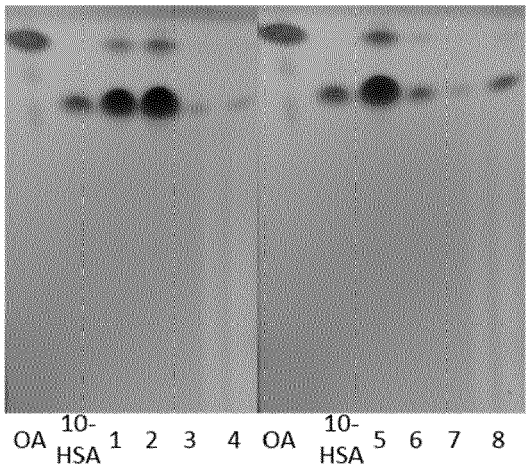


Figure 9:

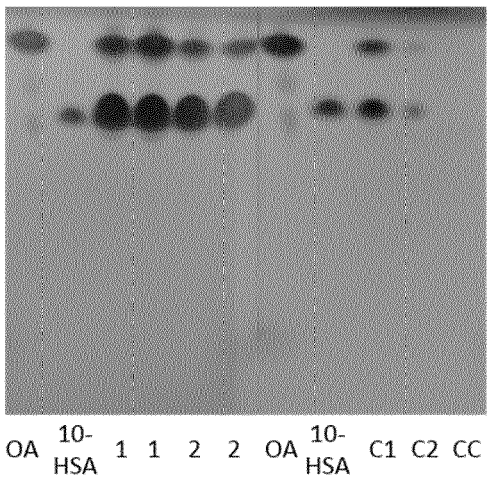


Figure 10:

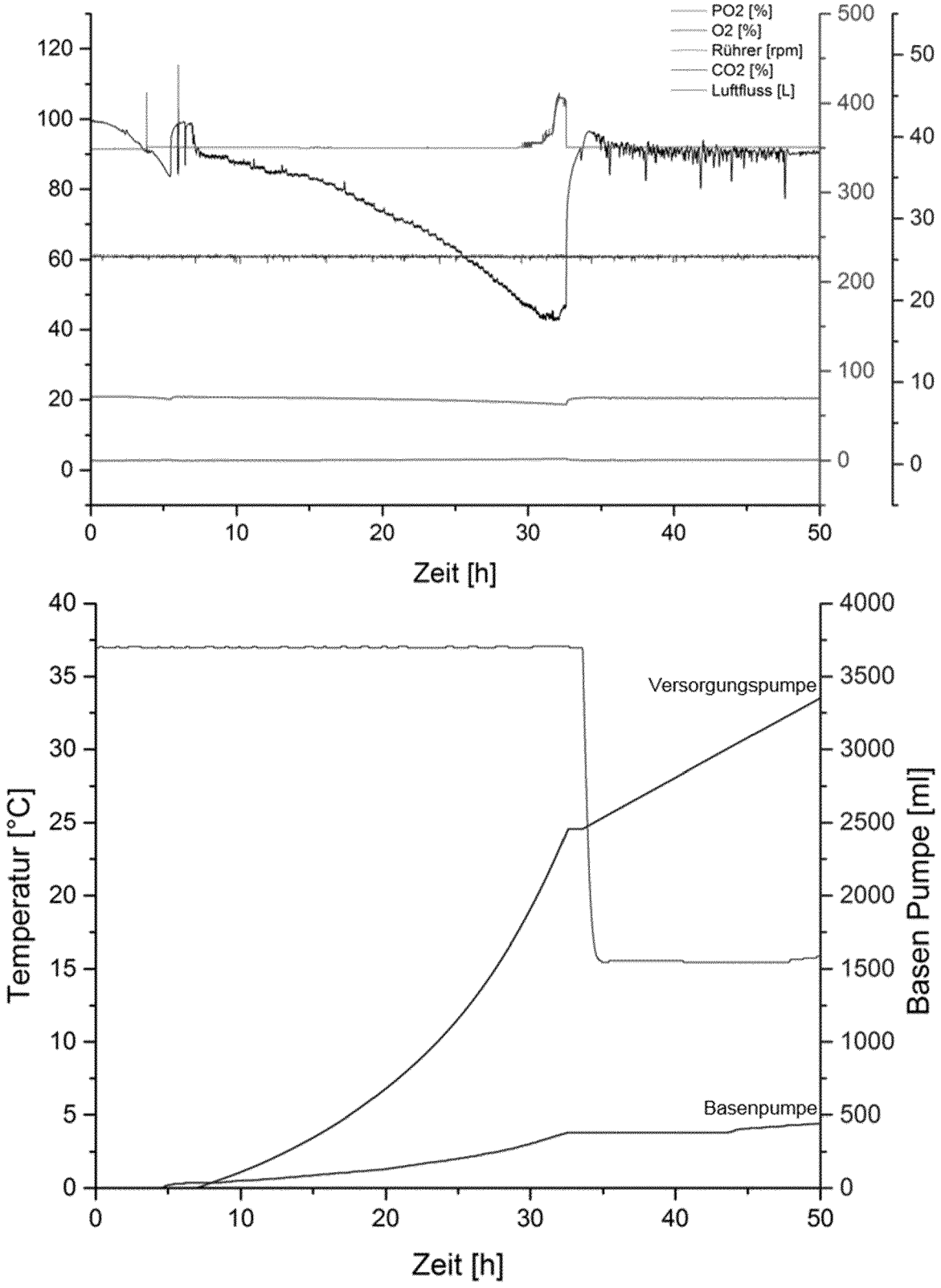

PO2 [%]
O2 [%]
Rührer [rpm]
CO2 [%]
Luftfluss [L]
Zeit [h]
Temperatur [°C]
Basen Pumpe [ml]
Versorgungspumpe
Basenpumpe
Zeit [h]

Figure 11:

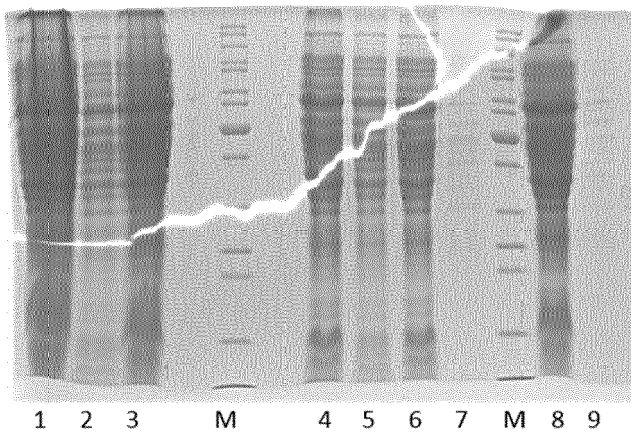


Figure 12:

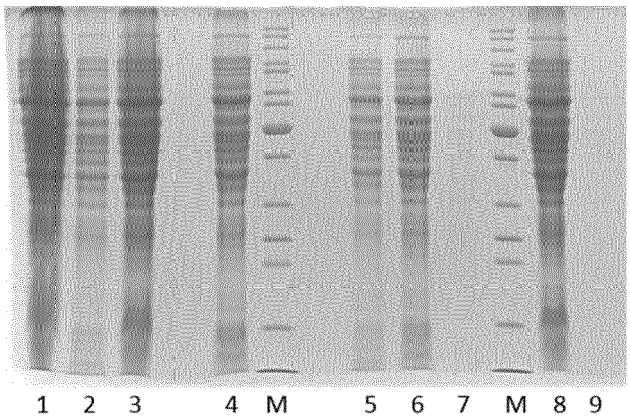


Figure 13:

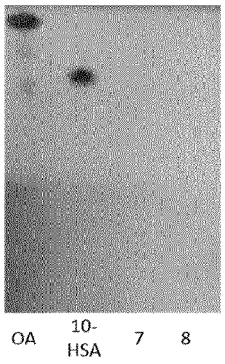


Figure 14:

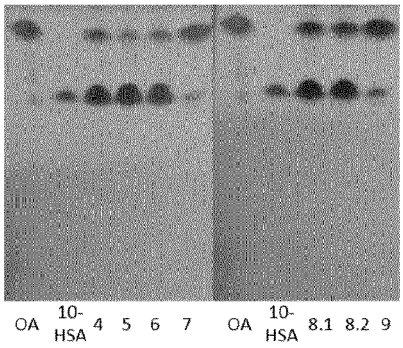


Figure 15:

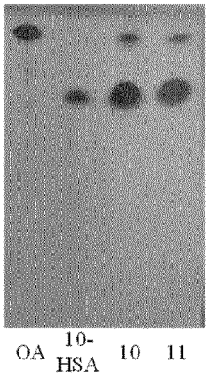


Figure 16:

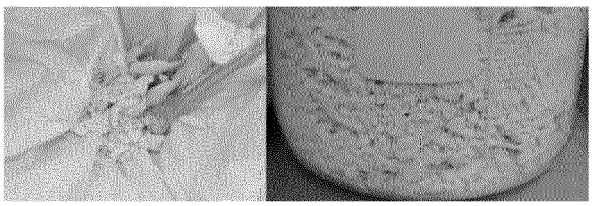


Figure 17:

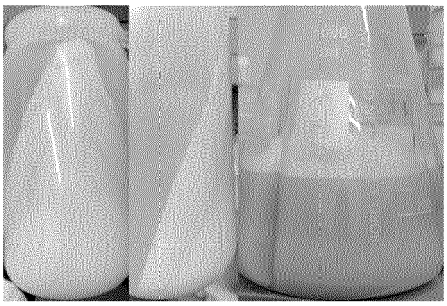
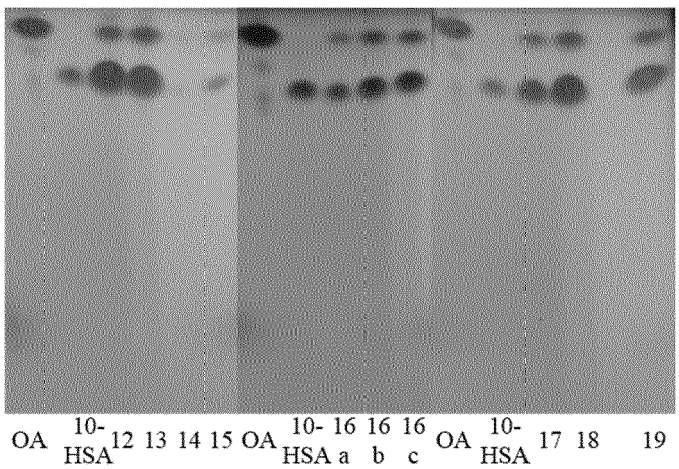


Figure 18:



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EUROPEAN SEARCH REPORT

Application Number
EP 17 19 4095

DOCUMENTS CONSIDERED TO BE RELEVANT

Category	Citation of document with indication, where appropriate, of relevant passages	Relevant to claim	CLASSIFICATION OF THE APPLICATION (IPC)
X	KANG ET AL: "Comparison of biochemical properties of the original and newly identified oleate hydratases from Stenotrophomonas maltophilia", APPLIED AND ENVIRONMENTAL MICROBIOLOGY, vol. 83, May 2017 (2017-05), pages 1-11, XP002775732,	17	INV. C12P7/42
Y	* See page 1 (Abstract), page 3 (Table 2), and page 7 (Figure 4) *	1-16	
X	DEMMING ET AL: "Optimized reaction conditions enable the hydration of non-natural substrates by the oleate hydratase from Elizabethkingia meningoseptica", CHEMCATCHEM, vol. 9, 31 January 2017 (2017-01-31), pages 758-766, XP002775733,	17	
Y	* See pages 758-759 (Abstract and Introduction) *	1-16	
X	SCHMID ET AL: "Biocatalytic study of novel oleate hydratases", JOURNAL OF MOLECULAR CATALYSIS B: ENZYMATIC, vol. 133, 2016, pages S243-S249, XP002775768,	17	TECHNICAL FIELDS SEARCHED (IPC) C12P
Y	* See page S248 (Table 1); online publication in Jan 2017 *	1-16	
X	KOPPIREDDI ET AL: "Combined biocatalytic and chemical transformations of oleic acid to omega-hydroxynonanoic acid and alpha-omega-nonanedioic acid", ADVANCED SYNTHESIS & CATALYSIS, vol. 358, 2016, pages 3084-3092, XP002775735,	17	
Y	* See page 3085 (Scheme 1) *	1-16	

-/--

The present search report has been drawn up for all claims

Place of search	Date of completion of the search	Examiner
Berlin	20 November 2017	Korsner, Sven-Erik

CATEGORY OF CITED DOCUMENTS

X : particularly relevant if taken alone
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O : non-written disclosure
P : intermediate document

T : theory or principle underlying the invention
E : earlier patent document, but published on, or after the filing date
D : document cited in the application
L : document cited for other reasons

& : member of the same patent family, corresponding document

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EUROPEAN SEARCH REPORT

Application Number
EP 17 19 4095

DOCUMENTS CONSIDERED TO BE RELEVANT

Category	Citation of document with indication, where appropriate, of relevant passages	Relevant to claim	CLASSIFICATION OF THE APPLICATION (IPC)
X	TODEA ET AL: "Increase of stability of oleate hydratase by appropriate immobilization technique and conditions", JOURNAL OF MOLECULAR CATALYSIS B: ENZYMATIC, vol. 119, 2015, pages 40-47, XP055270326,	17	
Y	* See pages 40-41 (Abstract/Immobilization and Introduction) *	1-16	
X	O'CONNELL: "Identification and characterization of an oleate hydratase-encoding gene from Bifidobacterium breve", BIOENGINEERED, vol. 4, 2013, pages 313-321, XP002775736,	17	
Y	* See page 316 (Figure 2 and Discussion) *	1-16	
X,D	KIM ET AL: "Production of 10-hydroxystearic acid from oleic acid and olive oil hydrolyzate by an oleate hydratase from Lysinibacillus fusiformis", APPLIED MICROBIOLOGY AND BIOTECHNOLOGY, vol. 95, 2012, pages 929-937, XP035090899,	17	TECHNICAL FIELDS SEARCHED (IPC)
Y	* See page 929 (Abstract) and pages 935-937 (Discussion) *	1-16	
X	HUDSON ET AL: "Conversion of oleic acid to 10-hydroxystearic acid by two species of ruminal bacteria", APPLIED MICROBIOLOGY AND BIOTECHNOLOGY, vol. 44, 1995, pages 1-6, XP035173261,	17	
Y	* See page 1 (Abstract/filtration) *	1-16	
X	WO 2016/151115 A1 (STICHTING DIENST LANDBOUWKUNDIG ONDERZOEK & TECHNISCHE UNI. DELFT) 29 September 2016 (2016-09-29)	17	
Y	* See Figure 4/triolein *	1-16	

-/--

The present search report has been drawn up for all claims

Place of search	Date of completion of the search	Examiner
Berlin	20 November 2017	Korsner, Sven-Erik

CATEGORY OF CITED DOCUMENTS

X : particularly relevant if taken alone
Y : particularly relevant if combined with another document of the same category
A : technological background
O : non-written disclosure
P : intermediate document

T : theory or principle underlying the invention
E : earlier patent document, but published on, or after the filing date
D : document cited in the application
L : document cited for other reasons

& : member of the same patent family, corresponding document

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EUROPEAN SEARCH REPORT

Application Number
EP 17 19 4095

DOCUMENTS CONSIDERED TO BE RELEVANT

Category	Citation of document with indication, where appropriate, of relevant passages	Relevant to claim	CLASSIFICATION OF THE APPLICATION (IPC)
L	KR 10-1 556 362 A (NOT AVAILABLE) * See [0017/S. maltophilia]; no reliable translation is available at present * -----		
L	DATABASE UniProt/SWISS-PROT [Online] October 2017 (2017-10), N.N.: XP002775789, Database accession no. B5XK69 * See the sequence; latest update in October 2017, earliest version from 2012 * -----		

TECHNICAL FIELDS SEARCHED (IPC)

The present search report has been drawn up for all claims

Place of search	Date of completion of the search	Examiner
Berlin	20 November 2017	Korsner, Sven-Erik

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